CANCER CELL BEHAVIOUR IN RESPONSE TO CHEMOTHERAPEUTICS — A STUDY OF DOCETAXELINDUCED INFLAMMATORY CYTOKINE PRODUCTION AND THE EFFECTS OF LIPOPOLYSACCHARIDES

by

Derek William Edwardson

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy (Ph.D.) in Biomolecular Sciences

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis Titre de la thèse	CANCER CELL behaviour in response to chemotherapeutics — a study of Docetaxel induced inflammatory Cytokine Production and the effects of Lipopolysaccharides
Name of Candidate Nom du candidat	Edwardson, Derek
Degree Diplôme	Doctor of Philosophy
Department/Program Département/Programme	Biomolecular Sciences
Date of Defence Date de la soutenance	mai 17, 2018

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. Amadeo Parissenti
(Co-Supervisor/Co-Directeur de thèse)

Dr. Thomas Kovala
(Co-Ssupervisor/Co-Directeur de thèse)

Dr. David MacLean
(Committee member/Membre du comité)

Dr. Carita Lanner

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
Monsieur David Lesbarrères
Dean, Faculty of Graduate Studies
Doyen, Faculté des études supérieures

Dr. Sheela Ramanathan
(External Examiner/Examinatrice externe)

Dr. Jeffrey
(Internal Examiner/Examinateur interne)

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

Abstract

The study of cancer is an ever-evolving discipline and since the 1950's it has been driven by fundamental scientific research using cultured human tumour cell lines isolated from human cancer patients. Over the years it has become evident that patients with cancer of the breast, ovaries, and several other tissues, often respond well to initial chemotherapy treatment, only to be left with tumours that have become resistant to the cytotoxic effects of chemotherapy. This has prompted decades of cellular and mouse-based studies to characterize the many biomolecular processes by which tumour cells in their microenvironments survive and reproduce in the presence of chemotherapy drugs. This dissertation discusses the role of cytokine production in chemotherapy drug efficacy both in the laboratory and the clinic. Cytokines are naturally released by healthy epithelial, endothelial, and immune cells to convey important messages to other cells and tissues of the body, driving immune responses upon recognition of pathogens or cellular damage. Cytokines have become increasingly considered for their roles in stalling or accelerating cancer progression as well as improving or limiting drug efficacy. In this thesis, we present primary research results that provide novel insight into the mechanism by which chemotherapy drugs induce inflammatory cytokine production and release from human tumour cells. We show that the semi-synthetic taxane derivative docetaxel, as well as other structurally distinct chemotherapy drugs, induce the release of the inflammatory cytokine TNF-α from breast and ovarian tumour cell lines. Constitutively increased production and release of TNF-α and CXCL1 from breast and ovarian tumour cells was also observed upon their selection for survival in increasing concentrations of docetaxel.

Docetaxel-resistant cells were less responsive to acute treatment with docetaxel than their drug-naive parental cell lines. These cells exhibited increased expression of the plasma membrane-bound drug-export protein, P-glycoprotein, which promotes the efflux of docetaxel and other drugs from tumour cells. Interestingly, restoration of drug into the docetaxel-resistant cells not only restored the drugs' cytotoxic effect but also the ability of the cells to respond to drug with increased TNF-α release. Current paradigms suggest that this response occurs through activation of the pathogen recognition receptor Toll-like receptor 4 (TLR4), involving direct interaction with docetaxel at the cell surface. This model appears inconsistent with our results showing that cellular drug accumulation is necessary for the response of increased TNF-α release to occur. We also show that the TLR4 agonist, lipopolysaccharides (LPS), causes increased production of TNF-α in the presence of docetaxel and increased docetaxel cytotoxicity for both wildtype and docetaxel-resistant MCF-7 tumour cells, representing a potential novel strategy to restore chemoresponsiveness in chemoresistant tumours.

Keywords

Cancer, chemotherapy, cytokines, taxanes, docetaxel, tumour necrosis factor (TNF-α), interleukin-8 (IL-8; CXCL8), GRO-α (MGSA; melanoma growth stimulatory activity; CXCL1), chemotherapy resistance, tumour progression, paclitaxel, doxorubicin, carboplatin, 5-fluorouracil (5-FU), breast cancer, MCF-7, MDA-MB-231, ovarian cancer, A2780, Lipopolysaccharides (LPS), Toll-like receptor 4 (TLR4)

Acknowledgments

I would like to acknowledge the hard work and perseverance of my committee members, colleagues, friends and family. If it were not for all of them I would not have made it here. I'd like to thank my supervisor Dr. Amadeo Parissenti for providing a graduate school experience full of opportunity and intrigue. I was honored to be a part of his lab group and to observe his expert approach to medical research. To my co-supervisor Dr. Tom Kovala, and committee members Dr. Carita Lanner, and Dr. Dave MacLean who challenged my ideas and made sure that I stayed on a good track. To Dr. Baoqing Guo for his practical guidance and good company in the lab. Much appreciation goes to my colleagues Simon Chewchuk and Jonathan Mapletoft for their friendship, support, and constructive advice. I would also like to acknowledge my wife Ashley for her patience and support at home, my siblings Brent and Amy for their lifelong support and encouragement, and my grandmother for her continued interest in my work. Finally, I must acknowledge my Mother and Father who, together as a team, have given me the most important things in life, including the ability to believe in myself.

Table of Contents

List of Figures

List of Tables

Abbreviations

µM	Micromolar
5-FU	5-fluorouracil
ABC	ATP-binding cassette transporter
ADAM-17	a disintegrin and metalloproteinase-17
AKR	aldo-keto reductase
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma-2
Bcl-XL	B-cell lymphoma-XL
bFGF	basic fibroblast growth factor
C/EBP	CCAAT-enhancer-binding protein
C5	complement component 5
CCL	(cysteine-cysteine) motif ligand
CCR	(cysteine-cysteine) motif receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
c-FLIP	cellular FLICE-like inhibitory protein
CXCL	(cysteine-x-cysteine) motif ligand
CXCR	(cysteine-x-cysteine) motif receptor
DAMP	damage-associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid

EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERK	extracellular signal-regulated kinases
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCSF	granulocyte colony-stimulating factor
GRO-α	growth regulated oncogene-α
HER	human epidermal growth factor receptor
HIV	human immunodeficiency virus
HMGB1	high mobility group box 1
HRP	horseradish peroxidase
IC50	inhibitory concentration 50
IFN	Interferon
IL-6	interleukin-6
JAK	janus kinases
LPS	Lipopolysaccharides
LPS-RS	lipopolysaccharides isolated from Rhodobacter Sphaeroides
MAP(s)	microtubule-associated protein(s)
MAPK	mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7
MDR1	multi-drug resistance protein 1
mM	Millimolar
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear transcription factor-κB
nM	Nanomolar
NQ01	quinone oxidoreductase
PAMP	pathogen-associated molecular pattern
PECAM-1	platelet and endothelial cell adhesion molecule-1
P-gp	P-glycoprotein
PI3K	phosphoinositide 3-kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SEM	standard error of the mean
siRNA	small (short) interfering ribonucleic acid
STAT	signal transducer and activator of transcription
TACE	tumour necrosis factor-alpha-converting enzyme
TICs	tumour-initiating cells
TLR	toll-like receptor
TNFR1	tumour-necrosis factor-α receptor
TNF-α	tumour-necrosis factor-α
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

Publications by the Author

Derived from Chapter 1 (section 1.2):

1) Edwardson D, Chewchuk S, Parissenti AM. Resistance to Anthracyclines and Taxanes in Breast Cancer. In: Breast Cancer Metastasis and Drug Resistance [Internet]. New York, NY: Springer New York; 2013 [cited 2017 May 22]. p. 227–47. Available from: http://link.springer.com/10.1007/978-1-4614-5647-6_13.

I contributed the majority of the writing for the publication, including sections 13.3 and 13.4, and all of the related subsections.

Derived from Chapter 1 (section 1.3):

2) Edwardson DW, Parissenti AM, Kovala AT. Chemotherapy and inflammatory cytokine signalling in cancer cells and the tumour microenvironment. Submitted to Springer.

I contributed the majority of the writing for the publication.

Derived from Chapters 2, 3, and 4:

3) Edwardson DW, Boudreau J, Mapletoft J, Lanner C, Kovala AT, Parissenti AM. Inflammatory cytokine production in tumor cells upon chemotherapy drug exposure or upon selection for drug resistance. PLoS One [Internet]. 2017 [cited 2017 Sep 19];12(9):e0183662. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28915246.

For this publication, I conducted experiments and generated data for all figures, except for figure panel 8B and figure panel 12A and 12B. I contributed all of the writing and played a primary role in addressing comments and critiques from the journal reviewers.

Not Derived from any particular section of the thesis:

4) Edwardson DW, Narendrula R, Chewchuk S, Mispel-Beyer K, Mapletoft JPJ, Parissenti AM. Role of Drug Metabolism in the Cytotoxicity and Clinical Efficacy of Anthracyclines. Curr Drug Metab [Internet]. 2015 Jan [cited 2016 Jan 7];16(6):412–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26321196.

I contributed the majority of the writing for this publication.

5) Chewchuk S, Boorman T, Edwardson D, Parissenti AM. Bile Acids Increase Doxorubicin Sensitivity in ABCC1-expressing Tumour Cells. Sci Rep [Internet]. 2018;8(1):5413. Available from: https://doi.org/10.1038/s41598-018-23496-y

I played a minor role in this publication, conducting one of the experiments using radioactively labeled docetaxel. I did not contribute any writing to the publication.

Chapter 1 - Literature Review

1.1 Overview

Cancer is a disease characterized by cell division that occurs without homeostatic control. Globally, this was the cause of death for more than 8 million people in 2012 alone, and was diagnosed in more than 14 million people, more than half in economically developed countries (1). Cancer diagnoses and deaths are projected to be 21.7 and 13 million (respectively) worldwide in the year 2030, considering the effects of population growth and aging (1). Lung cancer is the most common type of cancer in men worldwide, whereas prostate is the most common in men of developed countries. In women, breast cancer is the most common in both developed and developing countries and the leading cause of cancer deaths worldwide, with lung cancer being the leading cause of death for women of developing countries (1).

Depending on the tissue of origin, cancer is treated with one or more different therapies, including surgical removal of a tumour, chemotherapy, and radiation therapy. Chemotherapy and/or radiation can precede surgery (neoadjuvant treatment) or follow surgery (adjuvant treatment). Chemotherapy regimens that include drugs of the anthracycline and taxane classes are commonly used to treat patients with breast cancer, typically by intravenous infusions every two or three weeks. Although these regimens have shown clinical efficacy in slowing tumour growth, they often become less effective over time and can give rise to drug-

resistant tumours. Decades of research has shown that tumour cells can circumvent the toxic effects of anthracyclines and taxanes in a variety of ways, the majority of which are described in *section 1.2*.

The immune system and its various components are suggested to play a role in suppressing tumour formation and growth in healthy individuals. Our immune system communicates with many cell types in the body by way of organic molecules that are released by cells. These molecules can travel in the extracellular space or bloodstream, relaying messages to other cells to help coordinate their behaviour and maintain a state of homeostasis. It is becoming increasingly evident that a subset of these molecules, called inflammatory cytokines, are produced and also exploited by tumour cells, playing important roles in cancer progression and also in the efficacy of cancer treatment. *Section 1.3* provides a summary of the *in vitro* and *in vivo* research related to inflammatory cytokine production in response to chemotherapy treatment and the effects of inflammatory cytokines on tumour behaviour.

The mode by which taxanes and other chemotherapy drugs induce the production of various inflammatory cytokines has been the subject of much study, yet researchers lack a clear understanding of the mechanism(s) involved. Given the perceived importance of cytokine signalling in cancer treatment, a better understanding of how chemotherapy drugs induce cytokine production from tumour cells was sought in this study. Chapters 2, 3, and 4, describe the methodology, results, and discussion related to the experimental findings of this dissertation. In summary, we demonstrate that a variety of commonly used, yet structurally distinct, chemotherapy agents can promote the secretion of the inflammatory cytokine TNF-α

from breast and ovarian tumour cells in culture. In addition, we show that long-term selection of breast and ovarian tumour cells for survival in the presence of docetaxel results in the increased secretion of several inflammatory cytokines, including CXCL1 (GRO-α), CXCL8 (IL-8), and TNF-α. Interestingly, the inflammatory effects of bacteria-derived lipopolysaccharides and the taxane chemotherapy drug, docetaxel, were found to be distinct, despite existing reports that both agents elicit such effects through activation of toll-like receptor 4 (TLR4) at the cell surface. Activation of this receptor with lipopolysaccharides was found to result in increased sensitivity to docetaxel in both drug-naive and drug-resistant breast cancer cells, suggesting a synergy between the two agents and providing novel insight into approaches where TLR4 agonists currently in clinical use could potentially be used in augmenting chemotherapy efficacy against tumours in cancer patients.

1.2 Resistance to anthracyclines and taxanes in breast cancer

Breast cancer is the most common form of cancer among women worldwide, with incidence frequencies continuing to rise (2). This increasing incidence is generally attributed to prolonged life expectancy, urbanization and adoption of western lifestyles (2). Global statistics as of 2004 from the World Health Organization (WHO) estimate that breast cancer comprises roughly 16 % of all female cancers worldwide (2); of these, an estimated 519,000 women had succumbed to the disease in 2004 alone. The WHO estimates that a majority of these deaths occurred in developing countries, roughly 69 % (2). While incidence rates vary greatly worldwide they have been recorded to be as high as 99.4 in 100,000 women in North America. Moderate incidence rates have been recorded in eastern Europe, southern Africa, eastern Asia and South America, with the lowest incidence rates occurring in most African countries (2,3). As in the case with incidence rates, survival rates also vary greatly worldwide, ranging from 80 % in high income nations to less than 40 % in low income nations. These discrepancies are mostly attributed to availability of early detection and treatment methods (2,3).

Upon detection of disease that is contained within the breast, the primary treatment for breast cancer is typically surgical resection of the tumour with negative margins to prevent recurrence (4). This is because many patients with early-stage disease respond well to this treatment method. If the disease is sufficiently advanced but within the axilla, many adjuvant treatments exist for breast cancer, which include radiation therapy and a variety of chemotherapy regimens (4). Adjuvant therapy is generally designed to treat micrometastatic disease or breast cancer cells that have escaped the primary tumour but not yet established

identifiable metastases. Specific treatments differ depending on the nature of the tumour subtype (4). Locally advanced and inflammatory breast cancers, however, do not respond well to primary surgical techniques and are therefore deemed inoperable.

Table 1.1 - Anthracycline- and taxane-containing regimens for the treatment of breast cancer

Treatment Regimen	Chemotherapy agents used	Dose	Frequency	Cycles	References
TAC	Taxotere (Docetaxel)	75 mg/m^2 IV	Every 21 days	6	(5,6)
	Adriamycin (Doxorubicin)	50 mg/m^2 IV			
	Cyclophosphamide	500 mg/m^2 IV			
AC → T	Adriamycin	60 mg/m^2 IV	Every 21 days (14 days for dose dense)	4	(7,8)
	Cyclophosphamide	600 mg/m^2 IV			
	Followed by				
	Taxol (Paclitaxel)	175 mg/m^2 IV			
FEC 100	5-Fluorouracil	500 mg/m^2 IV	Every 21 days	6	(9)
	Epirubicin	100 mg/m^2 IV			
	Cyclophosphamide	500 mg/m^2 IV			
FAC	5-Fluorouracil	600 mg/m^2 IV	Every 21 days	4	(10,11)
	Adriamycin	60 mg/m^2 IV			
	Cyclophosphamide	600 mg/m^2 IV			
TC	Taxotere	75 mg/m^2 IV	Every 21 days	4	(12)
	Cyclophosphamide	600 mg/m^2 IV			
TCH	Taxotere	75 mg/m^2 IV	Every 21 days	6	(13)
	Carboplatin	AUC 6, IV			
	Trastuzumab (Herceptin)	4 mg/kg loading dose IV followed by 2 mg/kg/week x 18 then every 3 weeks x 12			

Neoadjuvant chemotherapy regimens are thus used as the first treatment for these breast cancers and typically include the anthracyclines and taxanes. These regimens include but are not limited to: TAC (Taxotere (Docetaxel), Adriamycin (Doxorubicin), and Cyclophosphamide) (5,6), AC → T (Adriamycin and Cyclophosphamide followed by Taxol) in both conventional and dose dense regimens (7,8), FEC 100 (5-fluorouracil, Epirubicin, Cyclophosphamide) (9), FAC (5-fluorouracil, Adriamycin, Cyclophosphamide) (10,11), TC (Taxotere, Cyclophosphamide) or TCH (Taxotere, Carboplatin, and Trastuzumab (Herceptin) for HER2-positive tumours (12) (see Table 1.1, adapted from (14)). Each of these chemotherapy drugs serves a different function in treatment. The taxanes (paclitaxel and docetaxel) function as anti-microtubule agents disrupting the cell's ability to divide during mitosis (5,6). The anthracyclines (doxorubicin and epirubicin) function as DNA damaging antibiotics (7,8). Cyclophosphamide is an alkylating agent, adding alkyl groups to the guanine bases of DNA, and Trastuzumab is a monoclonal antibody targeting and inhibiting the HER2 growth receptor present in some breast cancer types (10,11). Additionally, in early-stage breast cancer, adjuvant chemotherapy can play a critical role in the treatment of Estrogen Receptor (ER) positive cancers (15). Adjuvant therapy in these cases involves the use of compounds that target the estrogen signalling pathway, either through interfering with estrogen synthesis (aromatase inhibitors (Letrozole) or through selective estrogen receptor modulators (SERMs (tamoxifen)) (16).

Even with such available treatments, disease progression typically occurs in advanced breast cancers, likely due to the presence or development of chemotherapy-resistant tumours

(17). Some patients possess tumours that exhibit innate resistance to chemotherapy and do not respond to initial treatment (often referred to as ‘‘primary chemotherapy’’). These cancers are then typically treated with other chemotherapy drugs, if possible, or alternate treatments may become necessary, including surgery or radiation therapy (18). Other patients have tumours that initially respond or show partial response to the therapy. In such cases, a fraction or the majority of the tumour cell population is killed (18,19). The remaining drug-resistant cells, however, survive and continue to replicate, resulting in disease progression. Here we will explore some of the mechanisms associated with resistance to taxanes and anthracyclines in the treatment of breast cancers as well as some of the current work being done to manage patients with drug-resistant tumours.

1.2.1 Resistance to anthracyclines and anthracycline-based regimens in vitro

Anthracyclines are believed to be cytotoxic to tumour cells through three mechanisms. First, they intercalate between strands of DNA or RNA molecules and interfere with normal synthesis of these macromolecules in rapidly dividing cells (20). Second, they interfere with topoisomerase II, which is normally responsible for relaxing supercoiled DNA in order to facilitate DNA replication and transcription (21). Finally, anthracyclines cause cellular damage by facilitating the creation of iron-mediated oxygen free radicals (21).Many of the biochemical and cellular mechanisms of anthracycline resistance that have been identified to date have been obtained from *in vitro* studies and include drug export from tumour cells, and alterations in anthracycline metabolism, among others. *In vitro* mechanisms of anthracycline resistance were not within the scope of the thesis, however.

1.2.2 Mechanisms of resistance to taxanes in vitro

The taxanes block the growth of tumour cells by binding to microtubules and preventing their depolymerization, leading to mitotic catastrophe (22), multinucleation of cells, and the induction of apoptosis (23). One such apoptosis-inducing agent upregulated by the taxanes is the cytokine TNF-α (24). Like other chemotherapy drugs, the efficacy of taxane treatment is limited by a tumours' inherent or acquired ability to resist their killing action. Taxane resistance can be the product of a variety of alterations in cell behavior (23). A number of potential mechanisms of taxane resistance have been identified *in vitro*, including elevated expression of the ABC (ATP-binding cassette) family of drug transporters, alterations in microtubule structure and stability, inhibition of apoptosis, as well as the activation of some survival pathways.

1.2.2.1 Drug transporters and taxanes

One of the most studied mechanisms of drug resistance is the overexpression of the ABC transporters (25). ABC transporters are highly expressed in some tissues such as the intestinal epithelium and less differentiated cell types (26). They are associated with the membrane and actively transport a variety of molecules out of the cell (25). Among the ABC transporters is the permeability glycoprotein 1 (P-gp), also known as multi-drug resistance protein 1 (Mdr1) or Abcb1. It has been shown that P-gp contributes to taxane resistance in breast cancer cells *in vitro*, as its elevated expression correlates with low cytoplasmic concentration and decreased sensitivity to paclitaxel (27).

Breast tumour cell lines have been shown to develop P-gp mediated cross-resistance to drugs of the same class and in certain cases to drugs of different classes. Interestingly, breast

adenocarcinoma cells selected for resistance to doxorubicin showed several thousand-fold cross-resistance to both docetaxel and paclitaxel (28). An explanation for this may be that the anthracyclines first induce P-gp and that severe cross-resistance is observed because taxanes are a preferred substrate for P-gp over the anthracyclines (29). This observation may help to explain why patients were significantly less responsive to paclitaxel after late crossover from doxorubicin compared to treatment with doxorubicin after late crossover from paclitaxel (30). However, it is unclear whether P-gp or other ABC drug efflux transporters play a prominent role in clinical resistance to taxanes in breast cancer patients (See section 4.3 for more discussion related to P-gp and its potential roles in tumour response to chemotherapy).

1.2.2.2 Alterations in microtubule structure and stability

Microtubules are dynamic polymers essential to the cell that can undergo elongation and shrinking with the ability to interact laterally with one another (23). They are required for a variety of cellular processes including transportation of macromolecules and organelles, maintaining and changing structure of the cytoskeleton, and mitosis and cell division (31). Microtubules are made up of α and β-tubulin subunits, which can be in either a polymerized or dimer form (23). It is widely accepted that taxanes bind to β-tubulin in the polymerized form and increase polymer stability (23). In the case of cell division, the increased stability of β-tubulin leads to cell cycle arrest in mitosis (23) and eventually cell death. The β-tubulins are comprised of a variety of isotypes which vary at their C-termini (32). Molecular diversity among isotypes is accomplished by both the expression of distinct β-tubulin genes (32) and also post-translational modifications to β-tubulin gene products (33). Expression of certain β-tubulin

isotypes is tissue-specific, while other isotypes are more ubiquitously expressed (23). The functional specificity of different tubulin isotypes among tissues has yet to be determined (23).

Regardless of the β-tubulin isotype, polymers can form and the binding site for paclitaxel is only present in the case of the polymerized form of β-tubulin. Hence, it is suggested that selection for cancer cells in which the equilibrium between dimer and polymer has shifted toward dimer, could offer a survival advantage for a tumour that is treated with a microtubule-stabilizing agent such as paclitaxel (23).

Microtubule dynamics are also controlled by the differential expression of tubulin isotypes, mutations within tubulin genes, and also interactions with tubulin regulatory proteins. Tubulin regulatory proteins such as microtubule-associated proteins (MAPs) or stathmin interact with tubulin to promote polymerization or disassembly, respectively (23). Increased stathmin mRNA levels have been measured in breast carcinoma tissue from patients with more aggressive disease (34). It is also possible that post-translational modifications to tubulin such as phosphorylation, polyglutamylation, polyglycylation among others, may alter the binding of tubulin regulatory proteins, microtubule dynamics, and thus taxane efficacy (23).

1.2.2.2.1 Differential expression of specific β-tubulin isotypes

As mentioned, the tubulin isotype expressed in cells has an effect on the properties of polymer assembly and thus affects interactions with taxanes and microtubule dynamics. For example, microtubules assembled from βIII-tubulin are considerably less sensitive to the suppressive effects of paclitaxel on their dynamics, than microtubules assembled from βII-

tubulin (35). This suggests that selective expression of certain β-tubulin isotypes may affect the cellular sensitivity to taxanes.

A number of *in vitro* studies suggest a relationship between βIII-tubulin isotype levels and taxane resistance. For example, one study examined tubulin isotypes and mutations in paclitaxel-resistant cells by combined isoelectric focusing and mass spectrometry, and found that class III β-tubulin expression did, in fact, correlate with resistance to paclitaxel (36). Moreover, an association between class III β-tubulin expression and resistance to paclitaxel has been observed in a variety of human cancer cell lines of lung, ovarian, prostate and breast origin (37). However, another study showed that βI, βII, and βIII-tubulin levels were decreased and βIV-tubulin levels increased when MDA-MB-231 cells were selected for taxane resistance (38). Nevertheless, there is evidence of β-III tubulin's role in tumour resistance to taxanes in cancer patients (see 1.4.1), suggesting that differential expression of β-tubulin isotypes may be an important mechanism of taxane resistance in breast tumours.

1.2.2.2.2 Point mutations in tubulin

The binding of taxanes to β-tubulin subunits in microtubules can also be affected by mutations in genes coding for either β- or α-tubulin (39). These mutations can affect the sensitivity of cells to taxanes by causing a change in microtubule dynamics. It has been observed that cells with specific βI-tubulin mutations become resistant to paclitaxel *in vitro* (40), and that some paclitaxel-resistant cell lines depend on paclitaxel for survival (23). A potential explanation for this is that certain tubulin mutations shift the equilibrium in favor of the dimer form, such that cells harboring these mutations become hypersensitive to drugs that bind the

dimer form of tubulin such as colchicine and vinblastine (41). In some cases, the equilibrium is shifted to such an extent that the resulting lack of polymer stability compromises the cell's basic functions and thus paclitaxel's polymer stabilizing effects shift the polymer-dimer equilibrium in a more favorable direction, promoting survival (23).

Another form of taxane resistance can occur from a mutation in either α- or β-tubulin that alters the drug-binding site on β-tubulin polymers, such that it has less affinity for taxanes. *In vitro* reports of point mutations associated with taxane resistance in breast cancer cells have been reported, but in other studies, including clinical ones, no association between point mutations in tubulin genes and taxane resistance has been observed. For example, no mutations in β-tubulin genes were found when β-tubulin sequence information was compared between two docetaxel-resistant variants of the MDA-MB-231 and MCF-7 human breast adenocarcinoma cell lines and their drug-sensitive parental cell lines (38). A clinical study in 2003 also revealed that mutations in the class I β-tubulin gene did not predict response to paclitaxel in breast cancer patients (42). Thus, despite *in vitro* reports showing an association between β-tubulin mutations and taxane resistance, this association is not observed in breast cancer patients treated with taxanes.

1.2.2.3 Inhibition of apoptosis

The arrest in mitosis caused by taxane-binding to microtubules appears to promote the induction of apoptosis. The trigger for apoptosis is governed by the effects of taxanes on key apoptotic regulatory proteins. For example, it is believed that taxanes induce hyperphosphorylation of Bcl-2 and Bcl-x_L, which subsequently blocks their ability to bind to and

antagonize the apoptosis-inducers Bax and Bak (43,44). Bax and Bak are then free to dimerize and cause pore formation within the mitochondrial membrane, thus mediating apoptosis by the intrinsic apoptotic pathway (43–45). Taxanes also can cause Bax upregulation to promote apoptosis (43–45). It has also been suggested that paclitaxel can directly bind and sequester Bcl-2, a microtubule-independent mechanism of cell death (46).

The function of Bcl-2 is often regulated post-translationally by a variety of growth factor and cytokine signalling pathways (47). These pathways can drive Bcl-2 upregulation and induce paclitaxel resistance (48). For example, exposure to estrogen in estrogen-responsive breast adenocarcinoma cells (MCF-7) is associated with an increase in Bcl-2 levels and resistance to paclitaxel-induced apoptosis (49). Interestingly, one study found that induced recombinant ERα expression in ER-negative breast cancer cells caused resistance to paclitaxel by inhibiting apoptosis, while blocking ERα receptor activity in ER-positive breast cancer cells caused sensitization to paclitaxel (50). There is also clinical evidence that patients with ER-positive breast tumours are less responsive to paclitaxel than patients with ER-negative tumours (51–53).

Breast cancer cells selected for resistance to escalating doses of docetaxel were shown to have alterations in TNF-α signalling pathways. Specifically, the TNFRI receptor, which promotes cellular apoptosis, became downregulated upon resistance to docetaxel (24). This downregulation of TNFRI lead to increased activation of the transcription factor NF-κB, which promotes expression of anti-apoptotic survival genes such as c-FLIP (54) XIAP, and Bcl-$_{XL}$, which are known to cause chemotherapy resistance (55,56).

1.2.2.4 Activation of survival pathways

A cell's tendency to live or die is determined by the net balance of opposing death and survival pathways. Induction of survival pathways in breast cancer cells has been associated with resistance to taxanes. Several inflammatory cytokines have been shown to induce survival pathways in breast cancer cell lines, among other tumour types (See section 1.3). Taxane-resistant breast adenocarcinoma cells were shown to possess an amplified positive-feedback loop involving the TNF-α-dependent activation of NF-κB, which promoted expression of pro-survival genes. This involved the expression and secretion of cytokines, which complete the loop by way of autocrine signalling (24,57). Consistently, increased nuclear staining of NF-κB in tumours (indicative of activated NF-κB) has been shown to be associated with resistance to chemotherapy treatment with anthracycline- or taxane-containing regimens in breast cancer patients (58). Nevertheless, the true clinical relevance of such pathways in taxane resistance in breast cancer can only be determined through repeated clinical investigation.

1.2.3 Mechanisms of resistance to anthracyclines and taxanes in vivo

While providing significant insight into potential mechanisms of taxane or anthracycline resistance, the majority of the above *in vitro* studies fail to address important characteristics of human tumours that can impact on drug response and resistance. Such characteristics include their three-dimensional nature, the vasculature that provides nutrients and oxygen, and a complex tumour microenvironment comprised of surrounding stromal tissue, the extracellular matrix, and cells recruited by tumours (endothelial cells, fibroblasts, inflammatory cells of the immune system and pericytes). It is likely that some of these characteristics can account for the

lack of relevance of some *in vitro* drug resistance mechanisms in clinical studies. This tumour microenvironment creates the potential for cells within a tumour to be deprived of oxygen and nutrients, evade drug exposure, and exhibit a reduced proliferation rate, all of which could present a barrier to taxane or anthracycline cytotoxicity.

1.2.3.1 Changes in tubulin isoform expression

As mentioned previously, *in vitro* studies have shown that there may be a correlation between expression levels of specific tubulin isoforms and taxane resistance in breast cancer cells (36,37). Clinical studies appear to support such a view, as one study showed that breast cancer patients with high levels of class I and class III β-tubulin transcripts are less likely to respond to docetaxel than patients with the following levels of tubulin transcripts: class I-low/class III-low, class I-high/ class III low or class I-low/class III high (59). Also supporting this study, high tumour levels of tubulin β-I and β-III transcripts were found to correlate with clinical resistance to paclitaxel in advanced breast cancer (60). While these reports are compelling, further studies are required to assess whether tumour levels of tubulin β-I and β-III transcripts can serve as an effective biomarker of taxane resistance in multiple cohorts of breast cancer patients.

1.2.3.2 Interactions with stromal cells

Interactions between epithelial and stromal tissue play an important role in the function of healthy mammary glands (61) and mediate suppression of transformation to preneoplastic phenotypes (62). It has been suggested that cancer could be a physiological response to an abnormal stromal environment in some cases (63), as reviewed by Barcellos-Hoff and Medina

(64). In addition, stromal tissue can affect chemotherapy response through its tumour-supporting behavior (65).

Human cells communicate by secreting cytokines, chemokines, and growth factors that convey signals to nearby cells or travel through the bloodstream and affect more distal tissues. Activation of the innate immune response originates from the site of infection or inflammation, whereby signals are made available to components of the immune system, including monocytes, via the bloodstream. In breast cancer, signals originating from tumour or nearby stromal cells can strongly affect the host (patient) and may affect tumour response to chemotherapy. Accumulation of tumour-associated macrophages has been associated with poor prognosis in breast carcinoma, as they are suggested to exhibit a tumour-supporting phenotype in some cases, which can include secretion of cytokines that promote proliferation, angiogenesis, and metastasis (65).

It has recently been demonstrated that stromal gene expression can be an important factor in the clinical outcome of breast cancer patients treated with adjuvant chemotherapy (66). In this study, tumour stromal samples were classified as being from a patient with good, poor or bad outcome after assessment of clinical status post-treatment. Stromal overexpression of a specific set of immune-related genes, including T cell and natural killer cell markers, typical of a T_{H1} type immune response, was correlated with a good clinical outcome in patients (66). On the other hand, stroma from individuals in the poor-outcome group showed markers of hypoxia and angiogenesis, along with a decrease in chemokines that stimulate natural killer cell migration and mediate pro-survival signals in T-lymphocytes (66,67). In

another clinical study, mesenchymal/stromal gene expression signatures were shown to be useful in predicting resistance to neoadjuvant chemotherapy in breast cancer (68).

1.2.3.3 Nutrient deprivation, hypoxia, and acidity

Tumours are generally less vascularized than healthy tissue. As cells within a tumour reside farther from blood vessels, the level of nutrients falls and tumour cells in these areas tend to have decreased proliferation rates (69). It is suggested that since most anticancer drugs including taxanes and anthracyclines tend to be most toxic to rapidly dividing cells, slowly proliferating cells tend to be more drug-resistant (70). As nutrient levels are lower at distances further from vessels, so are pH and levels of molecular oxygen (71). Such hypoxic regions typically have increased expression of P-gp (72), which as mentioned, can cause taxane or anthracycline efflux from tumour cells. It has been suggested that anthracyclines may rely on superoxide formation as a means of cytotoxicity (73) and thus tumour cells in hypoxic regions may be less likely to suffer an attack of this nature (71).

Low pH in the tumour microenvironment is typical, as cancers often rely more heavily on glycolysis than normal tissues (74,75) and slower clearance of breakdown products (76–78). This can influence the cytotoxicity of anticancer drugs like doxorubicin, which are weakly basic. Protonation of such weak bases in acid environments could then result in decrease cellular drug uptake (78,79).

1.2.3.4 Drug penetration in tumours

Both taxanes and anthracyclines are administered intravenously and must cross capillary vessel walls to reach cancer cells. For cells in the interior of tumours, this requires extensive diffusion through multiple layers of tumour cells (referred to as ‘‘packing density’’) (70). By visualizing the location of doxorubicin through its natural fluorescence, it has been shown that high concentrations of doxorubicin are found within and around blood vessels, but concentrations of doxorubicin are considerably lower as the distance from the nearest blood vessel increases (80). It is suggested that the inability of both doxorubicin and epirubicin to penetrate deep into tumours may be the result of its sequestration in perinuclear endosomes and other organelles at the tumour surface or nearby host tissue (81). The contribution of P-glycoprotein-mediated drug-export in a patient tumour has also been suggested to be a factor affecting drug penetration into the tumour core. Radioactively labeled doxorubicin was shown to exhibit reduced penetration into a three-dimensional tumour cell model after P-gp inhibition (82).

1.2.3.5 Role of Tumour Initiating Cells in Anthracycline and Taxane Resistance

Solid tumours are generally heterogeneous, a product of their relatively high genetic instability. This results in tumours containing cells with a diversity of phenotypes, including rare cells exhibiting stem cell characteristics (quiescence, pluripotency, increased capacity for DNA repair) and both ABC transporter expression or dependence on surrounding stromal cells for survival (26). Such ‘‘stem cells’’ within tumours are referred to as tumour initiating cells (TICs)—

due to their ability to initiate tumour formation when injected into mice. Such cells may have significant relevance in taxane and anthracycline resistance in patients with breast cancer.

TICs have been identified in a variety of cancers including multiple myelomas (83), leukemias (84,85), colorectal (86), prostate (87), and hepatocellular carcinomas (88). Breast cancer TICs are defined by specific cell surface markers ($CD44^+/CD24^-/ALDH1^+$). Additionally, in many cases, breast cancer TICs have been shown to be dependent on developmental signalling pathways (89), particularly the Notch, WNT and Hedgehog pathways (89). Since TICs tend to possess properties similar to less differentiated cells, they may possess the ability to adapt to the adverse conditions caused by chemotherapy treatment (89,90). In addition, since TICs are relatively quiescent, they are less sensitive to chemotherapy agents targeting rapidly dividing cells, such as the taxanes or anthracyclines. They also may over-express ABC drug transporters, which are known to play a role in resistance to both taxanes and anthracyclines, as mentioned in previous sections (89,90). Nevertheless, there has been controversy about the cell surface markers that define breast TICs and which stem cell markers are correlated with chemo-resistance (90,91). ‘‘Basal-like’’ breast cancers are associated with poor patient prognosis and have many of the properties of TICs (92), but such cancers remain some of the most chemo-responsive tumours (93). Moreover, while clear subtypes of breast cancer have been identified through gene profiling studies (94) and while these subtypes differ in response to adjuvant chemotherapy (95), there are currently no pre-treatment genetic or protein biomarkers that can definitively distinguish between tumours that are responsive to anthracycline or taxane chemotherapy regimens and those that are not (94).

1.2.4 Management of breast cancer patients with drug-resistant tumours

Even if the appropriate biomarkers can be found to identify chemo-resistant tumours, the challenge of how to manage patients with such tumours remains. Typically, upon failure to respond to chemotherapy with anthracyclines and taxanes, treatment moves to other chemotherapy drugs in the adjuvant setting or to surgery and/or radiation therapy in the neoadjuvant setting. Strategies used to treat drug-resistant breast cancer involve the employment of drugs with mechanisms of action distinct from taxanes and anthracyclines, including capecitabine (96), navelbine, gemcitabine (97), and carboplatin.

Currently there has been little success in restoring drug sensitivity to patients whose tumours have acquired resistance to anthracyclines and taxanes (4). With increased knowledge of clinically relevant drug resistance mechanisms, it may be possible to interfere with these mechanisms to restore chemo-sensitivity. An early example of attempts to re-establish drug sensitivity by interfering with a drug resistance mechanism involves the employment of P-gp inhibitors in patients with chemotherapy-resistant tumours (98). Two such inhibitors, Verapamil and Tariquidar, were found to restore sensitivity to doxorubicin in drug-resistant cells (98), but had little effect on restoring clinical response to anthracycline- or taxane-containing chemotherapy regimens (99).

Another possible strategy to restore sensitivity to chemotherapy regimens in breast cancer patients involves the use of chloroquine. Chloroquine (Resochin) was originally developed as a drug to prevent malarial infections in humans (100). Its use has been expanded to include treatment for autoimmune disorders such as rheumatoid arthritis and recently as a

radio-sensitizing or chemo-sensitizing agent in cancer and HIV chemotherapy (101–104). In the case of cancer treatment, chloroquine is thought to act by inhibiting autophagic survival while activating apoptotic pathways (103,104). This occurs because chloroquine preferentially accumulates in lysosomes of the cells where the pH of the lysosomes traps the chloroquine (101). Additionally, chloroquine permeabilizes the lysosomes allowing for the release of lysosomal enzymes into the cytosol (101). Thus, chloroquine may sensitize tumour cells to radiotherapy or chemotherapy by interfering with autophagic survival pathways induced upon exposure to chemotherapy agents (101). Several clinical trials are currently under way to assess the efficacy of chloroquine as a possible tool to restore sensitivity to chemotherapy agents, such as the anthracyclines and taxanes. Research is also being performed on other autophagy inhibitors as sensitizing agents for chemo-resistant tumours.

Given that patient tumours vary in response to chemotherapy agents (both prior to and after previous rounds of chemotherapy), an additional approach to manage breast cancer patients would be to accurately assess tumour response to chemotherapy early in treatment, such that patients with non-responding tumours could be quickly switched to other downstream regimens such as surgery, radiation therapy, or other chemotherapy drugs. A recent study revealed that locally advanced breast cancer patients exhibiting a pathologic complete response to epirubicin/docetaxel chemotherapy post-treatment exhibited significant reductions in RNA integrity during chemotherapy (105). This ‘‘response biomarker’’ may be of particular value in patient management, if tumour response can be determined after one or two cycles of chemotherapy. The true value of this biomarker will only be determined through

additional studies involving the assessment of multiple cohorts of breast cancer patients at various cycles during chemotherapy treatment.

1.2.5 Summary

Anthracyclines and taxanes are powerful chemotherapy drugs used in the treatment of breast cancer, in particular for those patients that achieve a pathologic complete response to treatment with these agents. However, the majority of patients exhibit innate or acquired resistance to anthracycline- or taxane-containing regimens. While much has been learned from *in vitro* and *in vivo* studies on resistance to anthracyclines and taxanes in breast tumour cells, it appears likely that breast tumours evade the action of these agents through multiple mechanisms. Moreover, these mechanisms likely vary among patients and among the cell population within a given tumour. This makes it difficult to predict chemotherapy response and to identify a single small molecule that will block innate or acquired drug resistance. Nevertheless, significant advancements have been made in understanding the molecular diversity of breast cancers and their differential sensitivity to anthracyclines and taxanes. These tools are helping guide the oncologist in assessing a particular patient's risk of treatment failure. In addition to such *predictive* biomarkers, the development of *response* biomarkers may help confirm drug resistance early in treatment, such that non-responding patients can be moved more rapidly to alternate and potentially more beneficial treatments. The development of agents to prevent or combat resistance to anthracyclines and taxanes in select or multiple cohorts of breast cancer patients would help further improve the therapeutic benefit to patients with breast cancer. Given that the majority of patients do not receive a survival benefit

from adjuvant or neoadjuvant chemotherapy with anthracycline and taxanes (106,107), there is still significant and challenging work to be done.

1.3 Inflammatory cytokine signalling in tumours and tumour-associated tissues, and its potential implications in cancer chemotherapy

As discussed, resistance to chemotherapy drugs is a major problem in clinical oncology. Solid tumours from a variety of tissues, along with haematological neoplasms, can exhibit or acquire drug-resistant phenotypes. It has been suspected that these phenotypes, in many cancer patients, are driven by extracellular signalling between tumour cells, and also between tumour and immune cells. Further, it appears that inflammatory pathways can drive not only resistance to chemotherapy drugs but also cancer progression and metastasis. The following review describes the current state of knowledge related to cytokine signalling pathways and their implications in the treatment of various types of cancer.

1.3.1 Inflammatory signalling is an important part of our physiological response to pathogen recognition or cell damage

Of the trillions of cells that make up the human body, there are roughly 200 different types (108), which together make up the tissues, organs, and organ systems. Each cell type expresses a unique set of genes, while co-ordinating its behaviour with other cells by sending and receiving extracellular signalling molecules. These molecules can take on a variety of forms, some of which are released as relatively small chemical messengers derived from cholesterol (including the steroid hormones), and others that are multimeric proteins consisting of thousands of amino acids that can be either released as soluble entities or presented to receptors on the cell membrane. The effect of the signalling molecule depends on its molecular

structure, the presence of other signalling molecules in its surroundings, and the nature of the cell receiving it. Some of these molecules can act on more than one type of receptor and likewise, some receptors can be activated by more than one type of signalling molecule.

Along with the capacity to co-ordinate the activities of different tissues through cell signal transduction, the human body is equipped with a remarkable ability to detect and mitigate threats to homeostasis such as an invasion by a bacterial, viral, or fungal pathogen. Cellular receptors on a variety of human cell types are able to recognize distinct molecular patterns that are prevalent in pathogenic microorganisms (109). One such molecular pattern, which comprises the majority of the outer leaflet of the lipid membrane of Gram-negative bacteria, is called lipopolysaccharides (LPS). LPS provide bacteria with a protective barrier against toxic compounds, such as antibiotics, and allows them to survive in harsh environments (110). LPS are recognized as a danger signal to many types of eukaryotic cells, including human tissue.

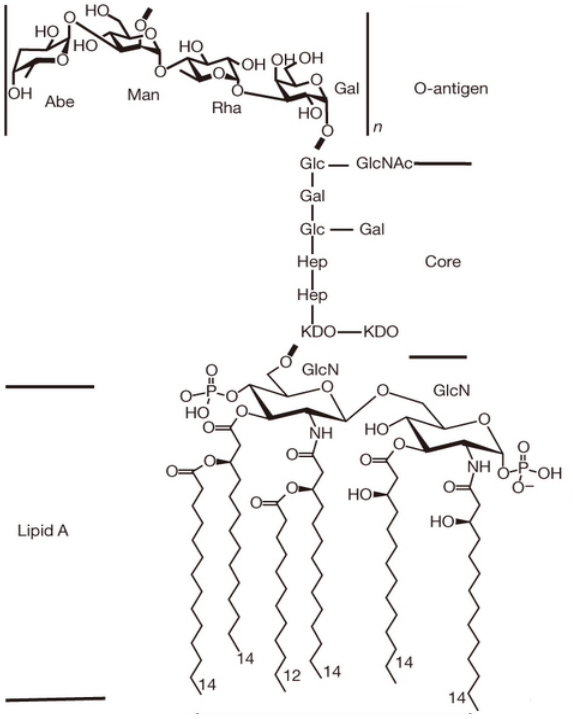

Figure 2.1 - Molecular structure of LPS - adapted from Dong et al. (111)

As depicted in figure 2.1, the general structure of LPS is comprised of lipid and sugar components, some of which are found to be more variable in nature than others. The lipid A component of LPS anchors it to the outer plasma membrane of bacteria (112). It is conserved relative to the O-antigen and core components and it is sufficient for inducing the pathophysiological effects caused when LPS is released by dying bacteria (113). Although the Lipid A structure is conserved, variations do exist among bacterial species. Some variations give rise to forms of LPS that are not immunogenic, while other forms even show antagonistic effects. For example, the penta-acylated form of LPS produced by *Rhodobacter Spheroides*

binds to the human receptor involved in LPS recognition and inhibits hexa-acylated (immunogenic) forms of LPS from provoking a response (114).

In multicellular organisms, a family of cellular receptors called the toll-like receptors (TLRs) are one of several families of protein receptors that specialize in the recognition of pathogen-associated molecular patterns (PAMPs). For example, binding of LPS to TLR4 (a TLR family member) results in receptor activation at the cell membrane surface, the heterodimerization of intracellular adaptor proteins and the activation of a variety of transcription factors that influence gene expression. Exposure of LPS to cells expressing TLR4 activates downstream pathway components and leads to the release of inflammatory cytokines (115–119). Inflammatory cytokines are part of the broader family of cytokine proteins that are released from many different cell types and regulate a variety of important processes in humans, including the activity and movement of leukocytes, nociception (pain), and inflammation. Inflammation, in particular, is the result of a local increase in blood flow, triggered by cytokine release, which presents as pain, localized heat, redness, swelling, and in extreme cases loss of function (120).

Inflammatory cytokines activate cells of the immune system, such as lymphoid and myeloid cells, to help maintain homeostasis during exposure to a pathogen. In humans, the immune system has two distinct components, the innate and adaptive immune responses. The recognition of a PAMP will first trigger an innate immune response involving cells with a specialized function (primarily macrophages and neutrophils), which can internalize and kill microbes that have breached the epithelial barrier, and also produce a variety of antimicrobial

compounds (121). The innate immune system, in some form or another, has coevolved with microbes, serving to protect all multicellular organisms from infection. Generally, innate responses are fast-acting measures but lack the specificity of adaptive (specific) responses, which take more time to develop (121). An adaptive immune response requires the phagocytosis of a pathogen-specific antigen, which is then presented to a T-lymphocyte (T-cell), stimulating its proliferation, and differentiation into a more activated state that can recognize and destroy foreign cells that possess the specified antigen (121).

As previously mentioned, toll-like receptors are known to play a role in the recognition of PAMPs but they're also involved in the recognition of one or more *endogenous* signals (122,123), called 'alarmins'. Alarmins are intracellular proteins, nucleic acids, or small molecules that are passively released into the extracellular environment during the disintegration of cells caused by mechanical force, excessive heat, cold, acids, bases, or cytotoxic poisons (124) and they have been found to play important roles in informing the host of tissue damage and the subsequent stimulation of reparative measures. They can also be actively released by immune cells through the endoplasmic reticulum/Golgi complex or other specialized secretion pathway(s). Among other functions, alarmins are recognized by cells of the innate immune system, promoting their chemotactic movement from the blood stream to the site of inflammation, and permitting adaptive immune responses (124), a function also shared by many inflammatory cytokines. PAMPs (exogenous) along with alarmins (endogenous) are considered subsets of a larger group of immunomodulators called damage-associated molecular patterns (DAMPs). Growing evidence shows that both can elicit similar

physiological responses, resulting in activation of cells that specialize in tissue repair and pathogen killing (124). Thus, it appears that at some level human cellular responses to *pathogen invasion* as well as *tissue damage* are inextricably linked. From an evolutionary perspective, this may be largely advantageous since tissue damage typically leaves an individual vulnerable to pathogen invasion, while pathogen invasion likely seldom occurs without some degree of tissue damage.

Among the alarmins, the high mobility group box 1 protein HMGB1 is one of the most well-studied. It is present in most human cells (125), where it functions as a nuclear cofactor in transcriptional regulation (126–129). During non-programmed cell death, such as secondary necrosis, cells will release HMGB1, which is able to induce an immune response. However, in the event of programmed cell death such as apoptosis, cells modify their chromatin so that HMGB1 remains bound and is not released into the extracellular environment (130). To a similar end, specific oxidation of cysteine residues on HMGB1 will prevent its interaction with TLR4/MD-2, blocking its ability to induce subsequent cytokine release from macrophages (128), and thus its inflammatory potential.

In summary, cytokines and alarmins can be released by a variety of human cell types and it has become evident that tumour cells are indeed a source of both. The following section will explore the documented roles of these extracellular signals, with an emphasis on inflammatory cytokines that are reportedly produced by tumours in response to treatment with chemotherapy drugs.

1.3.2 Chemotherapy-induced cytokine signalling in cancer cells and the tumour microenvironment

Cancer is the result of a cell's acquisition of a variety of biological capabilities or 'hallmarks' as outlined by Hanahan and Weinberg (131). These include sustained proliferative signalling, the ability to evade growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and the ability to invade other tissue and metastasize (131). More recently, the ability to escape immune destruction has been recognized as another hallmark. It is suggested that genome instability and *inflammation* accelerates the acquisition of a variety of the above hallmarks (131). Inflammation, is a product of the body's response to tissue damage or pathogen invasion. It is required for tissue repair and host defense, but prolonged inflammation can often be the cause for disease. In a cancer patient, it is often unclear whether inflammation plays a protective or deleterious role in disease progression. Chemotherapy drugs can suppress tumour growth but also induce inflammatory pathways in tumour cells, which has been shown experimentally to support tumour progression or, in other cases, encourage an anti-tumour immune response. Thus, with the goal of better understanding the context under which each of these possible outcomes occurs, recent progress exploring chemotherapy-induced inflammatory cytokine production and its effects on the tumour microenvironment will be reviewed.

For decades, inflammatory cytokines have been the topic of extensive study in oncology, implicated as promoters or in some cases inhibitors of tumour cell proliferation. They also are known to have effects on angiogenesis, metastasis, and another malignant cell behaviour.

Research has shown that several therapeutic agents used in the treatment of cancer can stimulate the production of several inflammatory cytokines from both cells of the immune system as well as the tumour. The extracellular space proximal to a tumour contains a repertoire of recruited, apparently normal cells, collectively referred to as the tumour microenvironment, which can contribute to progression of disease. Originating from either the tumour or other cells within its microenvironment, inflammatory cytokines in the tumour microenvironment play a major role in cancer behaviour (131).

The taxane class of chemotherapy drugs, including docetaxel and paclitaxel, are commonly used in the treatment of breast cancer, among other forms of the disease. Taxanes bind to β-tubulin where they exert their cytotoxic effects on tumour cells through the stabilization of microtubules and the resulting arrest in mitosis (132). Among the first documented studies of inflammatory cytokine production in response to treatment with chemotherapeutic drugs were reports of murine macrophages that increased their production of TNF-α and interleukin-1 after exposure to paclitaxel (133). It was later suggested that this action of paclitaxel is distinct from its cytotoxic activity (134). Figure 2.2 depicts the molecular structure of the most widely used taxanes, docetaxel and paclitaxel.

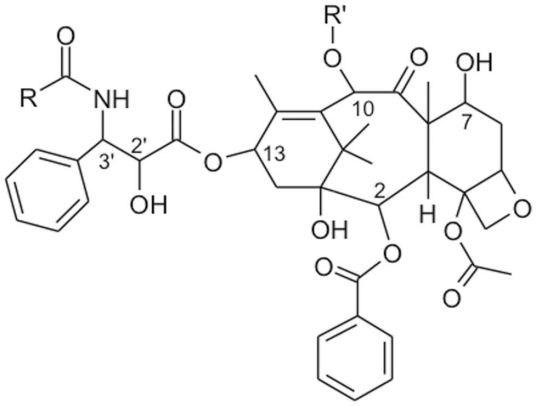

Figure 2.2 - Molecular structure of docetaxel and paclitaxel.

Docetaxel: R=tert-Butyl-O, R'=H; Paclitaxel: R=Phenyl, R'=Acetyl; adapted from (135)

Since then, the ability of taxanes, along with other drug families, to induce inflammatory cytokine production has been observed, in some cases from immune cells, and in other cases from tumour cell lines. Most cytokines act closely to where they are produced, often on the same cell from which they were released, which is called *autocrine* action. In other cases, cytokines produced by one cell act on another in a *paracrine* fashion. Some cytokines such as TNF-α can have important local effects, but also distant or systemic effects, referred to as *endocrine* function (121). The autocrine and paracrine actions of a variety of inflammatory cytokines have been shown to affect tumour behaviour. The table below presents a variety of studies related to chemotherapy-induced cytokine release.

Table 2.1 - Chemotherapy-induced release of a variety of inflammatory cytokines from various tumour and immune cell lines

Chemotherapy drug	Cytokine(s) induced	Cell type	Reference
Doxorubicin	MCP-1, IL-6, CXCL8	MCF-7	(57)
Camptothecin	IL-6	Lung cancer cell lines	(136)
Dacarbazine	CXCL8, VEGF	Melanoma	(137,138)
Cisplatin	IL-6	Lung cancer cell lines	(136)
Trastuzumab	EGF, IL-6	Breast cancer (BT-474) and gastric cancer (NCI-N87)	(139)
Docetaxel	C5/C5a, I-309, IFNγ, IL-1α, IL-1rα, RANTES	Prostate cancer cell line PC3	(140)
Docetaxel	IL-10	Docetaxel-resistant PC3 (prostate)	(140)
Docetaxel	CXCL1, RANTES	PC3 (prostate)/U937 (monocyte) mixed culture	(140)
Docetaxel	GCSF, IL-27	Docetaxel-resistant PC3 (prostate) / U937 (monocyte) mixed	(141)
Paclitaxel	TNF-α, IL-1α/β	Murine macrophages	(133)
Docetaxel	TNF-α	MCF-7 (breast), A2780 (ovarian) tumour cells	(24)
Paclitaxel	CXCL8	Ovarian tumour cells	(142)
Paclitaxel	CXCL8	Primary ovarian tumour cells	(143)
Paclitaxel	CXCL8	Human lung carcinoma	(144)
Oxaliplatin	CXCL8, CXCL1	Prostate cancer cell lines PC3, DU145	(145)
Doxorubicin	TNF-α	Breast epithelial cells (*in vivo* mouse)	(146)
Doxorubicin, cisplatin	IL-6, CXCL8, CCL2, CCL5, BFGF, G-CSF, VEGF	Tumour cells	(147)

1.3.3 Changes in tumour autocrine signalling

1.3.3.1 Tumour necrosis factor-α (TNF-α)

TNF-α is perhaps the most widely studied inflammatory cytokine, playing important roles in regulating both the innate and adaptive immune response pathways through its ability to affect a variety of cell types. It has been dubbed "a master regulator of leukocyte movement" (148), and recently has been shown to affect tumour cells directly through autocrine action.

We have shown that TNF-α is released from breast and ovarian tumour cells in response to treatment with chemotherapy drugs of the taxane class (24), as well as anthracyclines (doxorubicin), platinating agents (carboplatin), and nucleoside analogs (5-Fluoruracil) (149). It was found that when MCF-7 breast tumour cells were treated with a neutralizing antibody specific for TNF-α receptor 1 (TNFR1), the cells became less sensitive to the growth inhibitory effects of docetaxel (24). An explanation for this is that docetaxel-induced autocrine TNF-α signalling, through TNFR1, contributes to the drug's cytotoxic action on MCF-7 cells (24). Another study reported that addition of TNF-α, in recombinant form, potentiated the cytotoxic effect of paclitaxel in human SKOV3 ovarian cancer cells (150).

We also found that breast cancer cells selected for survival in increasing concentrations of docetaxel became resistant to the drug, while simultaneously increasing their production of TNF-α at intermediate selection doses (24). An investigation into the expression of TNF-α receptors revealed that concomitant with the acquisition of docetaxel resistance, there was a significant decrease in the level of TNFR1 protein levels, with no change in TNFR2 levels (24).

Thus, it is possible that while TNF-α signalling through TNFR1 activates a death pathway in the presence of docetaxel, abrogation of this pathway during acquisition of drug resistance shifts the effective balance of TNF-α signalling to the TNFR2 pathway, promoting survival. In support of this view, it was demonstrated that inhibition of TNFR2 signalling using a TNFR2-neutralizing antibody caused a sensitization of docetaxel-resistant breast tumour cells to docetaxel (24). Thus, it appears that depending on the relative levels of the two TNF-α receptors, TNF-α signalling can either increase or decrease the sensitivity of breast tumour cells to docetaxel. The above findings suggest that the autocrine role of TNF-α on tumour cells is context-dependent, depending upon the relative levels of TNF-α receptors present on target cells (24). As discussed later, the effects of drug-induced TNF-α release is also dependent upon the level of TNF-α in its soluble form (151), as well as its membrane-bound isoform (152).

1.3.3.2 CXCL8 (Interleukin-8 (IL-8))

Another inflammatory cytokine, CXCL8 has been studied for its potential role in cancer progression (153,154), metastasis (155), and drug resistance (156,157). CXCL8 plays many roles in immune responses and is released by a variety of immune cells, including monocytes, neutrophils, along with endothelial cells, mesothelial cells, and tumour cells (157). It possesses chemotactic activity and the ability to recruit neutrophils, basophils and T-cells to a site of immune activation (158–161). CXCL8 elicits its effects through two known cell-surface G protein-coupled receptors, CXCR1 and CXCR2 (IL-8RA and IL-8RB), which are expressed by most tumour cells (157,162) as well as endothelial cells (163).

Along with TNF-α, CXCL8 was found to be released from ovarian cancer cell lines in response to paclitaxel (142). Approximately fifty percent of human ovarian cancer cell lines responded to paclitaxel with increased CXCL8 production, a trend that extended to freshly explanted primary ovarian cancer cells (143). In responding cells, paclitaxel-induced CXCL8 expression required accumulation of drug within tumour cells and involved increased gene transcription (142). Also, paclitaxel-responsive elements located within the CXCL8 gene promoter were found to be necessary and sufficient for paclitaxel-induced CXCL8 production in responsive ovarian cancer cell lines (142).

Consistently, paclitaxel has been shown to upregulate CXCL8 synthesis in a subset of human lung carcinoma cell lines (144) and an independent study later showed that CXCL8 is a growth factor for both non-small and small cell lung carcinoma (164), specifically through the CXCL8 receptor CXCR1, but not through the other known receptor, CXCR2 (164).

Another study found that the level of basal CXCL8 production in A2780, SKOV3, and CAOV-3 ovarian cancer cells was negatively correlated with sensitivity to either paclitaxel or cisplatin, suggesting a connection between CXCL8 expression and drug resistance. In experiments where CXCL8 was over-expressed in poorly-expressing A2780 cells, cells exhibited increased resistance to both paclitaxel and cisplatin (157). In these studies, the authors suggested that CXCL8-induced drug resistance may be due to increased expression of the gene for the ABCB1 drug transporter *MDR-1*, which expresses P-glycoprotein (P-gp), as well as other genes. Similar findings from the same research group concluded that tumour IL-6 production, and subsequent autocrine signalling, resulted in increased resistance to paclitaxel and cisplatin

in ovarian cancer cells, through increased expression of multidrug-related genes, apoptosis inhibitory proteins, and activation of survival pathways (165).

In another study, the expression levels of IL-6 and CXCL8 were found to increase in MCF-7 breast cancer cells selected for resistance to paclitaxel. These cells were found not only to be resistant to paclitaxel, but also to doxorubicin and 5-fluorouracil, and exhibited increased expression of the ABC transporter P-gp (57). Furthermore, treatment with neutralizing antibodies or transfection with siRNAs targeting IL-6 and CXCL8 expression resulted in an increased sensitivity of the drug-resistant cells to both paclitaxel and doxorubicin. In support of a role for IL-6 and CXCL8 in drug resistance, the overexpression of both cytokines resulted in increased resistance to both paclitaxel and doxorubicin in drug-naive MCF-7 cells (57). The authors of these studies suggest that P-gp activity limits the accumulation of drug within cells, but that there are other factors contributing to drug resistance, since inhibition of the transporter with verapamil only partially restored sensitivity to drug (57). Critically speaking, without evidence that verapamil treatments completely restored accumulation of drug in the drug-resistant cells, it is possible that only partial inhibition of P-gp was achieved and this accounted for residual drug resistance. Thus, the drug-resistant phenotype could be mediated by P-gp and cellular export of drug, entirely. Regardless, these cytokines could indeed be drivers of P-gp expression or activity, and may promote the expression or activity of other survival pathways in tumour cells.

It is worth noting that CXCL8 transcript expression is consistently elevated upon acquisition of taxane-resistance in cancer cell lines (57,166), consistent with our own

observations in breast cancer cell lines selected for resistance to docetaxel that show an increase in CXCL8 release relative to their drug-naive parental cell line (149).

It is unclear whether CXCL8 plays a role in tumour drug resistance in a clinical setting. However, consistent with a role in drug resistance, elevated levels of CXCL8 in the tumours of patients with ovarian carcinoma were associated with poor prognosis in some studies (167,168).

Along with autocrine effects in both ovarian and breast cancer cells, reports suggest that CXCL8 may mediate resistance to oxaliplatin in metastatic prostate cancer cells (145). This study demonstrated an oxaliplatin-induced activation of NF-κB and increased gene transcription of CXCL8, CXCL1 and related receptor, CXCR2, in two androgen-independent prostate cancer cell lines. Inhibition of CXCR2 signalling resulted in abrogation of drug-induced NF-κB activation and increased oxaliplatin cytotoxicity (145). The table below presents studies that have demonstrated a role for one or more inflammatory cytokines, that have been shown to be upregulated by chemotherapy drug, in cells that are resistant to treatment through an autocrine signalling mechanism.

Table 2.2 - Cytokines identified as playing a role in resistance to cytotoxic agents via autocrine signalling and also induced by a chemotherapy agent

Cytokine(s)	Cytotoxic agent	Cell types involved	Tumour cell Targets*	Reference
Interleukin-6	Gemcitabine	Pancreatic cancer cells	FAK and ERK1/2	(169)
Interleukin-6	Trastuzumab	MCF-7 and SUM-159 HER2+ cells	IL-6R/STAT3/AKT/NF-κB	(170)
Interleukin-6	Trastuzumab	NCI-N87 gastric cancer cells	STAT3/Jagged-1/Notch	(171)
Interleukin-6, EGF	Trastuzumab	BT-474 (breast) and NCI-N87 (gastric)	STAT3	(139)
Interleukin-6	Dexamethasone	Various myeloma cell lines		(172)
Interleukin-6	Cisplatin	Non-small cell lung cancer cells (A549 and H157)	IL-6 gene transcription	(173)
Interleukin-6	Paclitaxel	SKOV3 and SKOV3/TR	IL-6 gene transcription / STAT3	(174)
Interleukin-6	Various anti-HER2 agents	ER-/HER2+ Breast cancer cells	IL-6/JAK/STAT3/ Calprotectin	(175)
Interleukin-6	Camptothecin, Cisplatin	Various lung cancer cell lines	Ataxia telangiectasia mutated / NF-κB	(136)
Interleukin-6	Doxorubicin, Vincristine, Paclitaxel	MCF-7	C/EBPβ, C/EBP, MDR-1	(176)
Interleukin-6	Tamoxifen	CAOV-3, SKOV3, ES-2, A2780	ER-α activity through MEK/ERK and PI3K/Akt	(177)
TNF-α	Docetaxel	Docetaxel-resistant MCF-7	TNFR2 / NF-κB	(24)

CXCL8	Cisplatin, Paclitaxel	A2780, SKOV3, CAOV-3	MEK/ERK and PI3K/Akt	(157)
Interleukin-6	Cisplatin, Paclitaxel	A2780, SKOV3, CAOV-3	MEK/ERK and PI3K/Akt	(165)
Interleukin-6	Paclitaxel, Doxorubicin	MCF-7	P-gp	(57)
CXCL8	Paclitaxel, Doxorubicin	MCF-7	P-gp	(57)
CXCL8	Oxaliplatin	Prostate cancer cell lines PC3, DU145	NF-κB	(145)

1.3.3.3 IL-6 (interleukin-6)

Autocrine signaling involving IL-6 and CXCL8 by triple-negative breast cancer cells has been found to be required for growth and survival, and the simultaneous knockdown of both genes was required to sensitize the cells to paclitaxel- or staurosporine-induced apoptosis (178). Trastuzumab (Herceptin) is a monoclonal antibody targeting the HER2 receptor and is commonly used to treat HER2-positive breast cancers. Treatment of HER2-positive breast cancer cells with trastuzumab induces the expression of IL-6, activating STAT3, which further upregulates IL-6 expression. This positive feedback loop has been shown to establish resistance to trastuzumab in breast (139,170) and gastric cancer (171). In HER2-positive cell lines, IL-6 activation of STAT3 resulted in increased S100A8/9 (calprotectin) levels, which activates proliferative and drug resistance pathways (175). Interleukin-6 has been shown, by a variety of groups, to drive drug resistance to chemotherapy drugs including gemcitabine, trastuzumab

(among other anti-HER2 agents), dexamethasone, cisplatin, camptothecin, doxorubicin, tamoxifen, as well as microtubule disruptors vincristine and paclitaxel in cancer originating from various tissue types (see table 2.2 for a list of studies). Although, it is among the most widely studied cytokine for its contribution to chemotherapy drug resistance (reviewed in (179,180)), IL-6 was not a major focus of this dissertation and will not be extensively discussed here.

1.3.3.4 CXCL1 (GRO-α)

CXCL1 plays a prominent role in the early stages of neutrophil recruitment during tissue inflammation (181) and activates the same cell surface receptor (CXCR2) as CXCL8. Production of CXCL1 in tumour cells was shown to occur *in vivo* after tumour-implanted mice were treated with doxorubicin, a study described later (146). CXCL1 has also been suggested to induce tumour cell proliferation in an autocrine fashion, since epithelial ovarian cancer cells (SKOV3 and OVCAR-3) over-expressing CXCL1 exhibited increased proliferation rates relative to wild-type cells (182). The shedding of EGF and activation of both EGFR and MAPK was required for the increased proliferative capacity, resulting from autocrine CXCL1 signalling through the G-protein-coupled receptor CXCR2 (182). As reviewed by Dhawan and Richmond, CXCL1 also has strong tumourigenic potential in melanocytes (183), with the ability to promote tumour formation in mice implanted with otherwise benign, immortalized melanocytes (184).

Thus, a variety of inflammatory cytokines have been characterized for their release and subsequent autocrine effects during chemotherapy treatment, mostly in an *in vitro* setting. Aside from TNF-α, it appears that most of the other inflammatory cytokines cited above are reported to have tumour-supporting autocrine effects. The relevance of chemotherapy-

induced cytokine pathways in tumour initiation and progression *in vivo* is, however, less clear. The net effect of changes in cytokine signalling pathways on tumour behaviour *in vivo* depends on signals from multiple cell types, involving autocrine and paracrine mechanisms that comprise a complex signalling network. The inherent complexity of paracrine signalling networks presents some difficulty for researchers. However, some groups have reported paracrine cytokine signalling loops involving tumour cells and their interactions with immune and endothelial cells within the tumour microenvironment.

1.3.4 Changes in tumour paracrine signalling

As mentioned, CXCL8 production in ovarian tumour cell lines was found to be triggered by paclitaxel (142), a frontline therapy used in the treatment of ovarian carcinoma. The biological significance of this phenomenon was assessed by the same group using an *in vivo* mouse model, whereby human ovarian cancer cells were transfected with an expression vector for CXCL8, injected into nude mice, and then tested for their ability to form a tumour (185). The group found that CXCL8 expression did not affect the growth of tumour cells *in vitro* but when the tumour cells were injected into mice, the growth of CXCL8-expressing tumour cells was significantly reduced compared to the control cells (185). Consistently, injection of a neutralizing antibody for CXCL8 caused an increase in tumour growth in the mice injected with CXCL8-expressing tumour cells. Interestingly, examination of mouse tissue near the injection site revealed dramatically elevated levels of neutrophils and macrophages in mice that were injected with CXCL8-expressing tumour cells. The group hypothesizes that the elevated immune cells within the tumour microenvironment may be due to CXCL8-dependent chemotaxis (185).

On the other hand, other groups found that overexpression of CXCL8 in ovarian carcinomas was found to be directly associated with tumour vascularity and cell proliferation (162), as well as metastasis in melanoma (186). In a clinical setting, immunohistochemical analysis of patient ovarian tumours revealed a positive correlation between CXCL8 levels and disease-specific mortality (156). This particular finding prompted a study involving orthotopically-implanted human ovarian tumour cell lines in mice. The study demonstrated that silencing of CXCL8, by administration of liposome-encapsulated CXCL8 siRNA, suppressed multiple tumourigenic properties, including angiogenesis and tumour cell invasiveness (156). The authors further provided data suggesting the production of CXCL8 by tumour cells promotes angiogenesis *in vivo*, as measured by elevated levels of the endothelial cell marker CD31 at the tumour (156), a cell marker known to increase proportionately to microvessel density. In addition, the above study showed that CXCL8 also induced MMP expression, which was associated with increased invasiveness of tumour cells (156). It is interesting to note that an *in vitro* assessment revealed that CXCL8 had no effect on HeyA8 and SKOV3ip cell line proliferation (156). This suggests that although CXCL8 autocrine signalling may promote invasiveness via MMP induction, its effect on tumour cell proliferation *in vivo* is through effects on endothelial cells and angiogenesis-dependent mechanisms. Although not discussed by the authors, immunohistochemical analysis of implanted tumours in the study revealed a detectable elevation in CXCL8 expression from mice treated with docetaxel and CXCL8 siRNA, relative to those treated with CXCL8 siRNA alone (156). Docetaxel treatment, however, did not result in changes in serum CXCL8 levels.

In a clinical setting, it remains unclear whether chemotherapy treatment induces cytokine release from tumours. Among several cytokines examined, blood levels of IL-6, CXCL8, and MCP-1 were found to decrease in patients with epithelial ovarian cancer following administration of steroids, but then significantly increased 24 hours after the administration of paclitaxel (187). The levels of these cytokines were also found to be constitutively elevated in peritoneal fluid, at concentrations two to three logs greater than those found in serum (187). Elevated levels of IL-6 and CXCL8 were found in patients with ovarian cancer relative to healthy controls, and high levels of these cytokines were found to be associated with a poor immediate response to paclitaxel (187). It was found in another study that IL-6 and CXCL8, among six cytokines tested, increased slightly in the blood serum of patients given paclitaxel for the treatment of non-metastatic breast cancer (188). However, the source of systemic inflammatory cytokines could be a variety of tissues, including peripheral blood leukocytes, tumour stroma, or tumour cells among other cell types, and it is unclear whether increased cytokine production in a tumour would be detected in the blood serum.

Clinical research has demonstrated what appears to be an intricate link between chemo-resistance and metastasis in cancer (189,190). A potential explanation for this is highlighted in a recent study describing a paracrine signalling network involving endothelial, myeloid, and breast tumour cells, whereby the alarmin (chemokine) S100A8/9, as well as inflammatory cytokines CXCL1 and TNF-α, play key roles in the interactions between tumour and healthy host cells (146). These studies used a mouse model to show that chemotherapy agents such as doxorubicin, paclitaxel, and cyclophosphamide, although toxic to tumour cells, are capable of

inducing TNF-α production in endothelial cells. This in turn, causes increased expression of chemokines CXCL1 and CXCL2 from tumour cells, which recruit CXCR2-expressing (CD11b+Gr1+) myeloid cells to the tumour (146). Recruited myeloid cells, in turn, release the alarmin heterodimer S100A8/S100A9, which promotes lung metastasis as well as breast cancer cell survival (146). The tumour-promoting effect of this heterodimer was shown to be mediated by ERK1/2 and p70S6K activation in tumour cells, as inhibition of these kinases was found to increase tumour sensitivity to doxorubicin (146). The table below summarizes the studies discussed here, which are related to the effects of tumour-derived cytokines on the tumour microenvironment.

Table 2.3 - Mouse studies related to the effects of tumour-derived cytokine

Effects on Tumour Microenvironment	**Tumour Cell line (Origin)**	**Mouse Model (Tumour location)**	**Reference**
CXCL8 attracted predominantly neutrophils (Gr1+) and some monocytes to tumour, suppressing tumour growth	OVCA 420, 429 (human ovarian cancer)	Female BALB/c-nu/nu mice (lower back)	(185)
CXCL8 contributed to angiogenesis, promoting tumour cell proliferation	HeyA8, SKOV3ip1, SKOV3ip.TR (human ovarian carcinoma)	Female athymic nude mice (NCr-*nu*) (intraperitoneal)	(156)
CXCL1/2 attracted myeloid cells (CD11b+Gr1+) to tumour, promoting tumour cell drug resistance and lung metastasis	LM2-4175 (Lung cancer metastasis from human breast cancer)	PyMT-F, PyMT-B (C57BL/6) (mammary fat pad)	(146)
TNF-alpha attracted myeloid cells to tumour and caused their differentiation to myeloid/endothelial phenotype, increasing angiogenesis and tumour progression	B-16 (mouse melanoma), LLC (mouse lung cancer), PyMT-derived (mouse breast cancer)	NOD/SCID, C57BL/6, FVB/nJ (mammary fat pad and/or flank)	(151)
membrane-bound TNF-alpha was cytotoxic to tumour-associated myeloid cells, suppressing tumour growth	LLC, B-16	C57BL/6 (subcutaneous)	(152)

While TNF-α is an important factor in cancer progression, its role appears to be quite dependent on the nature of the tumour and on the tumour microenvironment. Along with its well established effects on endothelial cells (191), more recent studies have shown a role for TNF-α, which affects tumour behaviour via its effect on tumour-associated myeloid cells. One study showed that "although high doses of TNF-α have antitumour activity" (151), TNF-α

expressed endogenously by tumours can cause differentiation of tumour-associated myeloid cells, resulting in a sub-population that expresses the vascular endothelial growth factor receptor (VEGFR2/Flk-1). Furthermore, this myeloid-endothelial subpopulation can support tumour progression in a variety of ways. In nude mice, significant increases in microvessel density (as measured by PECAM-1/CD31 immunoreactivity) were observed in the microenvironment of murine melanoma (B-16), murine lung (LLC) and murine breast (Py-mT) tumour cells upon transfection with an expression plasmid housing TNF-α cDNA, causing overexpression of the protein. Plasmids lacking the TNF-α cDNA were without effect. Consistent with this view, overexpression of TNF-α prevented necrosis in B16 and LLC tumour cells (151). Moreover, increased tumour cell proliferation was observed in TNF-α-overexpressing B-16 and Py-mT tumours, as quantified by Ki-67 immunoreactivity (151). Similar to the CXCL8 studies mentioned earlier (156), these tumour-supporting effects of TNF-α were not likely due to autocrine action, since overexpression of TNF-α did not significantly affect growth or survival of tumour cells in culture (151). While TNF-α overexpression from tumours gave rise to significantly larger CD11b+/F4-80+ myeloid cell populations expressing either VEGFR2 or VE-cadherin, these trends were not observed in TNFR1/2 double-knockout mice, thus confirming that TNF-α signalling through at least one TNF-α receptor was required for recruitment of the tumour-supporting myeloid cell population (151).

It has been suggested that TNF-α “orchestrates the interplay” between tumour and myeloid cell tissue, and that this interplay is important for tumour growth and metastasis (152). This cytokine is first produced as a membrane-bound protein before it is cleaved by matrix

metalloproteases and released from the cell membrane (192). Studies have exemplified a role for TNF-α in its membrane-bound form that is distinct from that of its soluble isoform. An *in vivo* study using mice with implanted lung or melanoma tumour cell lines revealed that the expression of the membrane-bound form of TNF-α (mTNF-α) in these cells, *exclusively,* caused a significant *reduction* in the tumour-associated myeloid-monocyte lineage (anti-ER-HR3, CD11b, F4/80), among tumour-associated T cells (anti-CD3), B cells (anti-B220b), and neutrophils (anti-Ly6G). More specifically, the study concluded that mTNF-α imposes a cytotoxic effect on myeloid cells, via the generation of intracellular reactive oxygen species. This in turn *prevents* the tumour-supporting behaviour of myeloid cells and results in reduced tumour growth (152). Similarly, a distinction between the effects of mTNF-α and the soluble form, sTNF-α, is highlighted in an *in vivo* study that examined the potential association between relative levels of TNF-α isoforms and patient outcome (193). Using gene expression data from 442 lung adenocarcinoma patients, the investigators found that higher tumour expression of TNF-α converting enzyme (TACE/ADAM-17), a matrix metalloprotease that cleaves membrane-bound TNF-α and releases the soluble isoform, was associated with lower overall survival (193). Also, those tumours with high TNF-α expression and low TACE levels (indicating a high mTNF-α:sTNF-α ratio) had longer survival times than patients with high TNF-α expression and high TACE (indicating a low mTNF-α:sTNF-α ratio) (193), consistent with the mouse model studies.

Certain chemotherapy agents have been reported to induce *immunogenic* cell death in tumour cells and growing evidence supports the idea that successful treatment of solid tumours *in vivo* with specific chemotherapy agents involves drug-induced signals originating

from tumour cells that promote tumour destruction by the host immune system (194–197). During chemotherapy administration a certain portion of the tumour cell population will succumb to treatment, thereby releasing damage-associated molecular patterns (DAMPs) that will provoke a response in nearby antigen-presenting cells. For example, one study (195) reported that HMGB1, released from tumours, binds TLR4 on the cell surface of nearby dendritic cells, activating a dendritic cell pathway. This pathway, delays the degradation of tumour-specific antigen that has been taken up by the dendritic cell, permitting more frequent tumour-specific antigen presentation to lymphocytes. This, in turn, facilitates an adaptive immune response that targets the tumour (195). Other DAMPs released from tumour cells, such as ATP, recruit myeloid cells to tumours, stimulating myeloid cell differentiation, and the phagocytosis of tumour antigens (198), thus promoting a tumour-targeted adaptive immune response. The cancer treatment regimen, whether ionizing radiation or chemotherapy, dictates the molecular signals released from dying tumour cells and the related immune response that is initiated, and this response has a significant affect on the clinical outcome (199). Given the above observations, there is little doubt that *inflammatory cytokines*, along with DAMPs, play major roles within the tumour microenvironment that, in turn, affect anti-tumour immune responses and clinical outcome.

1.3.5 Summary

In summary, chemotherapy drugs can induce the expression and release of inflammatory cytokines CXCL1, CXCL8, TNF-α, and IL-6 among others, from tumour cells of

various origins. Many of these cytokines can affect tumour behaviour through autocrine signalling, causing increased resistance to the chemotherapy drugs (24,57,157).

As discussed, *in vivo* mouse studies have shown that CXCL1 production by implanted tumour cells can be induced indirectly, through paracrine action requiring the release of TNF-α by endothelial cells, by doxorubicin treatment (146). Production of CXCL1, along with CXCL8 and TNF-α, by implanted tumours was shown to cause a number of changes to the tumour microenvironment, often resulting in disease progression, as summarized in Table 2.1.

It should be noted that mice do not normally produce CXCL8. However, they are arguably relevant models for such studies, since cells expressing both known CXCL8 receptors (CXCR1 and 2) are present in murine tissues and can thus be stimulated by CXCL8 produced by implanted human or murine tumours secreting human CXCL8. *In vivo* mouse studies have demonstrated that tumour production of CXCL8 can promote a variety of important changes to the tumour microenvironment, including recruitment of myeloid cells, and changes in the behaviour of tumour-associated stromal cells. The biological effect of CXCL8 release from tumours appears to be dependent upon the tumour context, either inhibiting tumour progression (185) or, in other cases, supporting a variety of malignant phenotypes, including increased tumour vascularization through its effects on endothelial cell function (156). Similarly, tumour-generated TNF-α can increase tumour vascularization through its ability to induce an endothelial phenotype in tumour-associated myeloid cells (151). Although increased vascularization reportedly increases tumour proliferation and progression in mouse tumour studies (151,156), it is possible that increased blood flow to tumours could result in improved

drug delivery to the tumour and improved drug efficacy. Consistent with all *in vivo* studies reviewed here, production of the inflammatory cytokines CXCL1, CXCL8 or TNF-α by tumour cells seems to affect tumour-associated myeloid cell behaviour. Whether these effects are in support of tumour progression (or protective against it) depends upon one or more confounding factors.

The immune system appears to have a duplicitous role in cancer. Whether it helps or hinders tumour progression depends upon the stimulus, be it tumour-derived and/or a response to therapy, and perhaps, most importantly, the underlying health of the patient and his or her immune system.

Changes triggered by certain chemotherapy drugs can have significant effects on tumour behaviour either directly through their cytotoxic effects on the tumour or through their indirect effects on non-malignant cell populations within the tumour microenvironment. Taking into account the chemotherapy regimens used to treat the patient, the potential inflammatory mediators produced, and their subsequent effects on cells in the tumour microenvironment, appears to be critical to personalize and improve the quality of care for cancer patients.

1.4 Hypothesis and objectives

1.4.1 Hypothesis

Despite the important role that inflammatory cytokines play in cancer progression and drug resistance, it is still unclear as to which biochemical pathways are required for their release from tumour cells in response to chemotherapy agents, such as those of the taxane family. The idea that taxanes can activate inflammatory pathways has been the focus of much study. This class of drug has often been shown to have 'LPS-mimetic' effects, as it has been reported that paclitaxel induces similar changes in gene expression to LPS (200). LPS, also referred to as bacterial endotoxins, are the major structural components of the outer cell wall of Gram-negative bacteria. They are believed to be the cause of severe sepsis in patients with Gram-negative bacterial infections and it is suggested that most of the adverse effects of LPS stem from their ability to trigger the endogenous production of inflammatory cytokines (201). Among the inflammatory cytokines, TNF-α is believed to be a primary mediator of sepsis, since direct infusion of animals with recombinant TNF-α produces most of the adverse events observed after LPS administration (202). The pathogen recognition receptor TLR4, is the primary receptor involved in LPS signalling. Activation of TLR4 by LPS has been shown to cause nuclear factor-κB (NF-κB)-mediated expression of TNF-α and CXCL8 (203), among other genes (204). Another member of the TLR family (TLR2) has also been shown to mediate LPS signalling (205), although to a lesser extent. Treatment of myeloid cells with LPS has been shown to result in the release of a variety of cytokines including TNF-α (119), CXCL8 (206), and CXCL1 (181). Each of these cytokines has been implicated in resistance to chemotherapy agents through

either autocrine (24,157) or paracrine (146) signalling mechanisms. Likewise, taxane-induced release of several inflammatory cytokines, from tumour cells as well as myeloid cells, is thought by many groups to require the *direct* activation of TLR4, at the cell membrane surface (119,200,207–210); however, clear evidence for this has yet to be demonstrated in tumour cells. It should also be noted that increased CXCL8 release was observed after taxane treatment of ovarian tumour cells that lack a response to LPS, and this required drug entry into the cell (142). Given these findings and the prior observations by Sprowl and colleagues (24), we hypothesize that:

(a) drug-induced TNF-α release is dose-dependent and can occur in response to acute exposure to a variety of chemotherapy agents in tumour cells originating from both the breast and the ovarian epithelium,

(b) docetaxel-induced TNF-α release is an active cellular response of live cells during treatment, and not a passive release from dead cells,

(c) the mechanism of docetaxel-induced TNF-α release is distinct from the effects of lipopolysaccharides (LPS), such that LPS stimulates TNF-α production in drug-resistant tumour cells and, in the presence of docetaxel, may augment docetaxel cytoxicity, and

(d) sustained exposure to docetaxel and selection for resistance to drug causes changes in the basal production levels of multiple inflammatory cytokines that have been implicated in drug resistance.

1.4.2 Objectives

The above-described literature shows that inflammatory cytokines, such as TNF-α, are produced by a variety of cell types in response to treatment with various chemotherapy agents, and affect tumour behaviour by acting on either the tumour itself or the tumour-associated stroma. Given the lack of a clear understanding of the mechanism of taxane-induced inflammatory cytokine production in human tumour cells, and the importance of TNF-α-mediated signalling in the acquisition of resistance to taxanes, this study sought to:

(a) provide further insight into the mechanisms by which docetaxel and LPS induce the release of TNF-α from tumour cells,

i) by determining the exposure time and drug concentration that will optimally induce the release of TNF-α from human breast and ovarian cancer cells,

ii) by assessing whether docetaxel-induced TNF-α release is merely a passive cellular process resulting from the loss of membrane integrity during cell death, or whether it is an active cellular response to drug exposure.

iii) by comparing and contrasting the inflammatory effect of docetaxel and LPS on human tumour cells, in terms of the inflammatory cytokines released and the receptors involved in the response to treatment with each of the agents,

(b) determine whether TNF-α release changes after exposure to a variety of chemotherapy agents that are commonly used and act in mechanistically distinct ways for the treatment of solid tumours, and

(c) assess whether prolonged exposure to docetaxel results in changes in the expression of TNF-α and other inflammatory cytokines that promote drug resistance,

i) by first characterizing the drug-resistant phenotype exhibited in MCF-7 and A2780 cell lines that were previously selected for survival in increasing concentrations of docetaxel, and

ii) by comparing basal cytokine release between drug-naive and drug-resistant cell lines.

Given that the treatment of solid tumours using chemotherapy regimens is often unsuccessful and that treatment can induce inflammatory cytokine production from tumour cells, which in some cases has been shown to drive resistance to chemotherapy cytotoxicity, a better understanding of the mechanism(s) involved in chemotherapy-induced cytokine production from tumour cells is a worthy goal. The general focus of this *in vitro* study is to improve our understanding of the effects of short and long-term exposure of tumour cells to docetaxel.

Chapter 2 - Materials and Methods

2.1 Cell culture

MCF-7 cells were obtained from the American Tissue Culture Collection (Virginia, USA), while A2780 cells were from the European Collection of Authenticated Cell Cultures (Salisbury, UK). Cells were cultured in DMEM (Thermo Fisher, Mississauga, ON) and RPMI (GE Healthcare Life Sciences, Mississauga, ON) media using Sarstedt and Corning T75 tissue culture flasks for MCF-7 and A2780 cells, respectively, in a 5% CO_2 atmosphere at 37 °C. Culture media was supplemented with 10% FBS (v/v) and 1% Penicillin and Streptomycin (v/v). At confluence, cells were washed with PBS, treated with 0.25% trypsin for resuspension after which 1 ml of complete media was added to inhibit trypsin activity. Cell suspensions were harvested by centrifugation at 650 x g for 7 minutes and resuspended in medium for counting. Docetaxel-resistant variants were generated from MCF-7 cells and A2780 cells as previously described by Guo et al. (28) and Armstrong et al. (211), respectively. MCF-7_{TXT7}, MCF-7_{TXT8}, MCF-7_{TXT9}, MCF-7_{TXT10}, MCF-7_{TXT11}, and MCF-7_{TXT12} cells are MCF-7 cell lines selected for survival in progressively increasing concentrations (doses) of docetaxel (also known as Taxotere® (TXT)). These doses (7 through 12) are 0.37, 1.1, 3.3, 5, 15, and 45 nM docetaxel, respectively. Likewise, $A2780_{DXL10}$, $A2780_{DXL11}$, and $A2780_{DXL12}$ cells represent a series of A2780 cell lines selected for survival in up to 1.97 nM (dose 10), 2.96 nM (dose 11), and 8.88 nM (dose 12) docetaxel, respectively. During the above selections for docetaxel resistance, equivalent cultures of MCF-7 and A2780 cells were passaged in the absence of drug to control for differences in cell properties and behavior

due to increased passage number. One of these "co-cultured control" cell lines at selection dose 10 for the MCF-7 cell line and at selection dose 12 for the A2780 cell line (MCF-7_{CC10} and $A2780_{CC12}$ cells, respectively) served as the drug-sensitive control cell line in experiments involving drug-resistant cell lines.

2.2 ELISA (enzyme-linked immunosorbent assay)

All ELISAs were completed using kits from R&D systems (Minneapolis, MN, USA), following the manufacturer's experimental protocol. Plates pre-coated with monoclonal antibody were purchased for the detection of the cytokines TNF-α, CXCL8, and CXCL1 (catalog numbers DTA00C, D8000C and DGR00, respectively). Equal numbers of cells were transferred to 10 cm culture plates and allowed to adhere overnight. After treatment for up to 96 hours, the culture media were collected and subjected to centrifugation at 875 x g to remove cell debris. The media supernatants were then transferred to a 50 ml Amicon tube equipped with a 3 kDa molecular-weight-cut-off filter (EMD Millipore, Etobicoke, ON; cat no. UFC900324) and subjected to centrifugation at 3273 x g. The volumes of media supernatants were measured and the samples stored at -80 °C. ELISA standards were prepared by serial dilution of a reconstituted lyophilized stock solution. After 2 hours of incubation at room temperature, the wells were rinsed with wash buffer. A horseradish peroxidase (HRP)-conjugated secondary antibody was then added to each well and the plate incubated for 1 hour at room temperature, followed by a rinse with wash buffer and addition of HRP-sensitive colourimetric substrate for 20 minutes. The absorbance of the samples at 450 nm and 540 nm was measured using a

Synergy H4 Hybrid spectrophotometer from BioTek® (Winooski, VT) and duplicate measurements were averaged for both samples and standards. After subtracting the absorbance at 540 nm from the absorbance at 450 nm absorbance values were likewise corrected for background absorbance of standard diluent (for standards) or concentrated tissue culture medium (for samples).

2.3 Cell counting and trypan blue staining

Following trypsinization and staining with 0.066% trypan blue (v/v), cells were counted in a hemocytometer (Improved Neubauer, Hausser Scientific, (Horsham, PA)) using a light microscope (Leica - 10x/0.22) with phase contrast filter. The average number of trypan-positive and trypan-negative cells within a 4x4 grid of 4 different fields was determined.

2.4 Reverse-transcription of mRNA and quantitative PCR (polymerase chain reaction)

Two million cells were transferred to 10 cm tissue culture dishes and grown for 30, 36, and 42 hours. RNA was isolated using a Qiagen RNeasy extraction kit (Qiagen, Inc., Toronto, ON). The cells were washed with PBS, and treated with 350 µl of RLT buffer containing 3.5 µl of β-mercaptoethanol. Cells were harvested using a cell-scraper, and the lysate was subjected to 4-5 passes through a 16-gauge needle. After transfer of the lysate to a microfuge tube, an equal volume of 70% ethanol was added and, after mixing, the mixture was transferred to a Qiagen mini-column and the RNA was purified as described in the manufacturer's protocol. The concentration of purified RNA was determined by measuring the absorbance at 260 nm in a

Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with a baseline correction for absorbance at 340 nm. A mass of 2 µg of purified RNA was diluted to generate a mixture of 15 µl, containing 2 µl of 10x DNase buffer, and 2 µl of DNase (1 unit/µl). After a 15-minute incubation at room temperature, 2 µl of 25 mM ethylenediaminetetraacetic acid (EDTA), 20 µl of T20 primers (20 ng/µl) and 16 µl of dNTP's (10 mM total) were added. Samples were incubated for 5 minutes at 65 °C after which 16 µl of 5x first-strand buffer and 8 µl of dithiothreitol (DTT) were added, with a subsequent incubation period of 2 minutes at 37 ^{o}C. Finally, 200 U of Moloney-murine leukemia virus (M-MLV) reverse-transcriptase was added and the mixture left for 2 hours at 37 ^{o}C to allow for reverse-transcription of mRNA to cDNA. Samples were then heated for 5 minutes at 95 ^{o}C to inactivate the transcriptase.

Quantitative real-time PCR reactions were performed in a 96-well plate using cDNA preparations. Standards for mRNA were generated using cDNA from an untreated sample originating from the same cell line as the other samples, to generate a standard curve. Five or more 2-fold serial dilutions were made starting with an initial 4-fold dilution in RNase-free water. Samples were prepared beginning with a 16-fold dilution (3 µl of sample added to 45 µl of RNase-free water). The primer pair for detection of TNF-α transcripts was 5'-CCT GCC CCA ATC CCT TTA TT-3' (forward) and 5'-CCC TAA GCC CCC AAT TCT CT-3' (reverse). A primer pair with sequences 5'-TCC ATC CGC AAT GTA AAA-3' (forward) and 5'GCT TCT CGC TCT GAC TCC AAA-3' (reverse) was also used to quantify expression of the S28 reference gene. All primers were purchased from Integrated DNA Technologies (Skokie, IL, United States). Triplicate

samples were prepared for qPCR with 5 µl per well of cDNA standards, including a no-template control containing only RNase-free water, or 5 µl per well of cDNA for unknown samples, to which was added 7.5 µl of 2 mM pooled forward and reverse primers and 12.5 µl per well of 1x SYBR Green. The plate was centrifuged for 1 minute at 650 x g in order to position all reagents at the bottom of each well. qPCR involved a hot start for 5 minutes at 95 °C, followed by 40 cycles consisting of: 30 seconds at 95 °C, 30 seconds at 53 °C, and 1 minute at 72 °C.

2.5 Flow cytometry

Cells (2 x 10^5 per well) were allowed to adhere overnight in 6-well plates. The cells were rinsed with PBS, resuspended with 500 µl of 0.25% trypsin, transferred to 1 ml microfuge tubes, combined with 500 µl of PBS, and centrifuged for 3 minutes at 300 x g. The pelleted cells were resuspended in a mixture of 20 µl of phycoerythrin-conjugated monoclonal antibody, specific for P-glycoprotein (P-gp), protected from light and left at room temperature for 30 minutes. Cells were centrifuged for 3 minutes at 300 x g, washed with PBS, resuspended in PBS and then analyzed with an FC500 flow cytometer (Beckman Coulter, Mississauga, ON) set to measure fluorescence using the FL2 filter for 10,000 events per sample with no gating. Mean fluorescence Intensity (MFI) values for the mouse anti-human P-gp antibody or for an IgG2β isotype control (BD Bioscience, catalog 557003 and 555743, respectively) were recorded and the MFI for the isotype control subtracted from the MFI for the P-gp-specific antibody to correct for non-specific binding.

2.6 Drug uptake measurements

Cells ($2x10^5$) were plated in 6-well plates and left to adhere overnight in the appropriate medium. Cells were then incubated with either 2.5 nM tritium-labeled docetaxel (^{3}H-TXT from American Radiolabeled Chemical, St. Louis, MO) or 100 nM Tariquidar (Med Chem Express, Monmouth Junction, NJ) or a combination thereof, with 5% CO_2 at 37 °C. After 12 hours, the media were removed, while the adhered cells were rinsed with 1 ml PBS, treated with 0.25% trypsin, and then placed in 5 ml of scintillation fluid. The radioactivity associated with the cells was then quantified using a Beckman LS 6000 IC scintillation counter (Mississauga, ON).

2.7 Cell protein extraction

Two million A2780 and MCF-7 cells were seeded onto 10 cm tissue culture plates and cultured for 24 and 48 hours, respectively. Protein extracts were prepared from these cells by lysis in 500 µl of chilled RIPA buffer (1% NP-40 (Sigma, Oakville, ON), 0.5% sodium deoxycholic acid (Sigma) and 1% SDS (Biorad, Mississauga, ON) in PBS) supplemented with 2 mM sodium orthovanadate (Sigma) and 1X protease inhibitor cocktail (Roche, Mississauga, ON). Cell lysates were passed through a 21-gauge needle 5 times, incubated for 30 minutes on ice, and then centrifuged at 15,000 x g for 30 min. The supernatants were divided into aliquots that were stored at -80°C. The protein concentration of extracts was quantified using the Pierce BCA protein assay kit (Thermo Fisher, Mississauga, ON). Alternatively, cells were extracted in 5X SDS-PAGE sample buffer to ensure complete dissolution of membranes for enhanced isolation of membrane-associated proteins.

2.8 Immunoblotting experiments

Lysates (36 µg of protein) were loaded onto a 10% polyacrylamide gel for electrophoresis, transferred to a nitrocellulose membrane using a Biorad Trans-blot® SD Semi-Dry Transfer Cell for 1hr at 12V. The membrane was blocked in 5% skim milk in TBST (0.24% Trizma® Base (Sigma, Oakville, ON), 0.8% NaCl (Thermo Fisher, Mississauga, ON), 0.1% Tween20® (Sigma) at pH 7.6) for 1 hour before incubation overnight at 4°C with a human TLR4 antibody (1:250, Santa Cruz, Dallas, TX), human MyD88 antibody (1:1000, Cell Signalling, Danvers, MA) or a GAPDH antibody (1:10,000, Santa Cruz). All primary antibodies were diluted in TBST supplemented with 5% BSA (Sigma). Membranes were washed with TBST 3 times for 5 minutes. TLR4 and MyD88 levels were detected using an HRP-conjugated goat anti-rabbit secondary antibody (1:10,000, Santa Cruz). The membranes were washed before incubation with ECL reagent (Santa Cruz) and exposure on CURIX ortho HTL-Plus film (AGFA Healthcare, Waterloo, ON). Densitometry was performed using the FluorChem FC3 apparatus and AlphaEaseFC 4.0 software.

2.9 Clonogenic assays

Cells were added to 6-well plates ($3x10^5$ cells) with 2 ml of media per well and left to adhere overnight. Each well received one of twelve different concentrations of docetaxel (including a 'no drug' control) and the cells incubated for 24 hours. After 24 hours the medium in each well was collected, while the adherent cells were resuspended using 0.25% trypsin. The floating and adherent cells from each well were combined, centrifuged at 650 x g for 7 minutes,

and resuspended in 300 µl of culture media. The cells were then added to 2.7 ml of methylcellulose (with 25% FBS v/v), and mixed thoroughly by vortexing. After incubating an hour to allow bubbles to escape, 1.2 ml of each cell suspension were transferred to a 6-well plate. The plates were then incubated for two weeks and photographs of 12 independently chosen fields were taken using a Leica light microscope with a Leica 4x/.10 objective lens. Viable colonies, defined as being greater than or equal to 4 mm (MCF-7) or 3 mm (A2780) in two perpendicular directions (as measured on a PowerPoint slide and printed 4 slides per 8'x11' page), were counted. The average colony count was then computed and divided by the average colony count for the untreated control to obtain the survival fraction. Survival fractions were then plotted against drug dose and a sigmoidal dose-response (survival) curve was fitted to 12 data points using GraphPad Prism software. The concentration that inhibited colony formation by 50% (IC_{50}) was determined for each dataset at the inflection point of the sigmoidal curve. Clonogenic assays using Tariquidar (Med Chem Express, Monmouth Junction, NJ) involved 24-hour co-administration of Tariquidar and docetaxel together, whereas clonogenic assays with LPS (Sigma, Oakville, ON) involved a 48-hour pre-treatment with LPS followed by a 24-hour docetaxel treatment. Tariquidar clonogenics used 3×10^5 MCF-7 cells and 2×10^5 A2780 cells, whereas LPS clonogenics used 2×10^5 and 1×10^5 cells for MCF-7 and A2780 cells, respectively.

Chapter 3 - Results

3.1 Characterization of docetaxel-induced TNF-α release

3.1.1 Assessing the effects of drug concentration and exposure time on TNF-α release

In order to provide further insight into the mechanism by which docetaxel induces the release of TNF-α from tumour cells, we tested the effects of drug concentration and time of exposure (figure 3.1), with the goal of determining the optimal treatment conditions for inducing increased TNF-α release from tumour cell lines that originated from the breast and ovarian epithelium, as specified in objective (a-i). We previously showed that MCF-7 breast and A2780 ovarian tumour cells exhibited increased production of TNF-α, 48 hours after treatment with 3 to 45 nM docetaxel (24). In our current study, we found that 2.5 nM docetaxel induced increasing TNF-α release up to 96 hours after addition to various cell cultures (figure 3.1A and B). A dose response curve was generated at the 72-hour time point for each cell line (figure 3.1C and D). We observed that both the concentration of drug and the exposure time are important factors affecting TNF-α release by docetaxel in both breast and ovarian cancer cell lines. It was also found that A2780 cells expressed considerably higher levels of TNF-α in response to docetaxel than MCF-7 cells, with the peak TNF-α concentrations greater than ten-fold above those observed in MCF-7 cells (figure 3.1C and D).

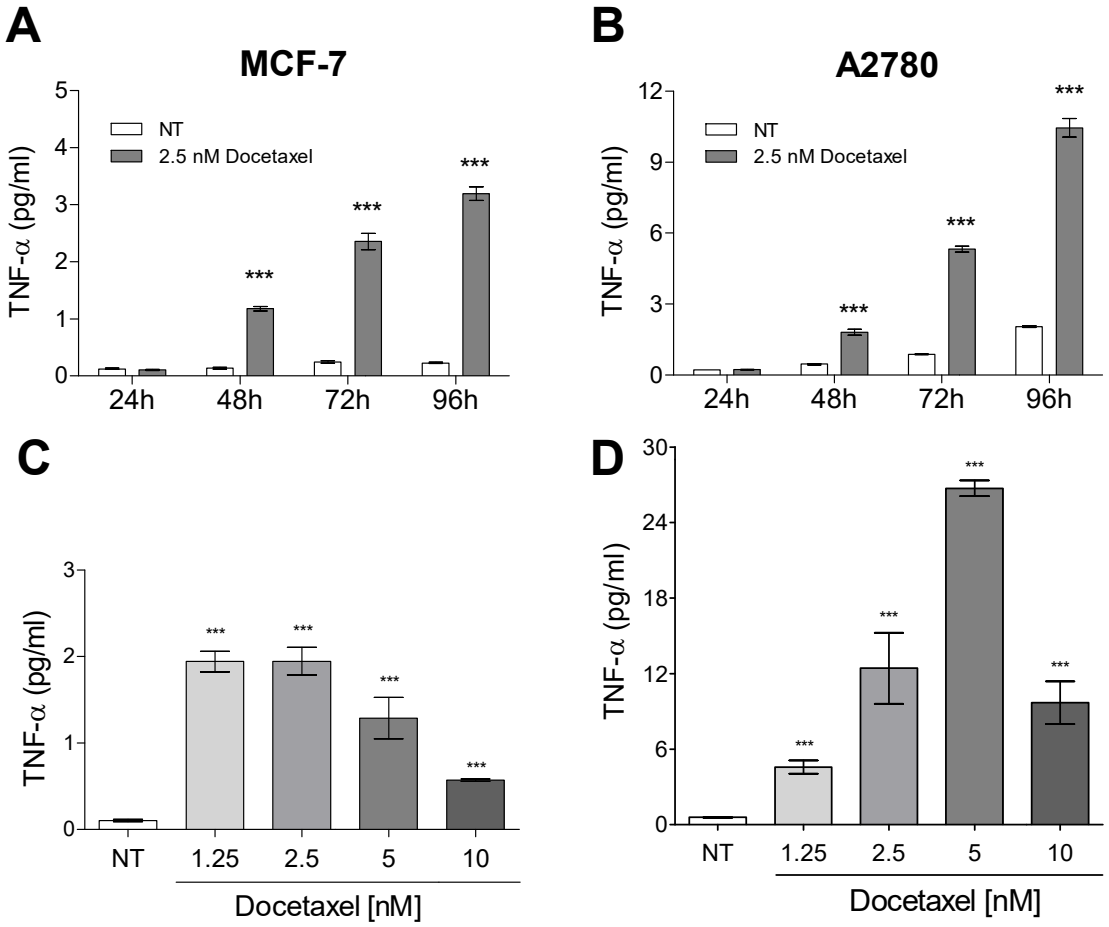

Figure 3.1 - Docetaxel-induced TNF-α levels in media of breast and ovarian tumour cells are dose- and time-dependent. A) and B) Effects of treatment time on TNF-α release from MCF-7 and A2780 cells after the addition of 2.5 nM docetaxel; C) and D) Effects of docetaxel concentration on TNF-α release from MCF-7 and A2780 cells after 72 hours. The data represents the mean of three replicates (+/- SEM). The significance of differences in secreted TNF-α levels between treated and untreated (NT) cells was assessed using a two-tailed T-test; *** $p<0.0001$

3.1.2 A variety of classes of chemotherapy drugs induce TNF-α release from breast and ovarian tumour cell lines

Members of several different classes of chemotherapy agents such as the taxanes (docetaxel and paclitaxel), the anthracycline doxorubicin, the platinating agent carboplatin, and the thymidine analog 5-Fluorouracil (5-FU) were used to treat various tumour cell lines for 72 hours at various concentrations. This experimentation was conducted to address objective (b), which is to determine whether TNF-α release changes after exposure to a variety of chemotherapy agents that are commonly used and act in mechanistically distinct ways for the treatment of solid tumours. The levels of TNF-α released into the medium were assessed using a TNF-α ELISA. Given that the concentration of docetaxel (2.5 nM) that optimally induced TNF-α release in MCF-7 cells at 72 hours was approximately five-fold higher than the IC_{50} for docetaxel, the concentrations used for testing the effects of other drugs were chosen relative to their published IC_{50} values in a previous study using MCF-7 cells (28). MCF-7 cells responded to docetaxel, paclitaxel, doxorubicin, and 5-FU with significant increases in TNF-α levels. Carboplatin was without effect. However, MDA-MB-231 and A2780 tumour cells responded to all of the agents with statistically significant increases in secreted TNF-α levels. Both MDA-MB-231 and A2780 cells secreted more TNF-α than MCF-7 cells. Overall, docetaxel was the chemotherapy drug with the greatest capacity to induce TNF-α release in the cell lines tested (figure 3.2A, B and C).

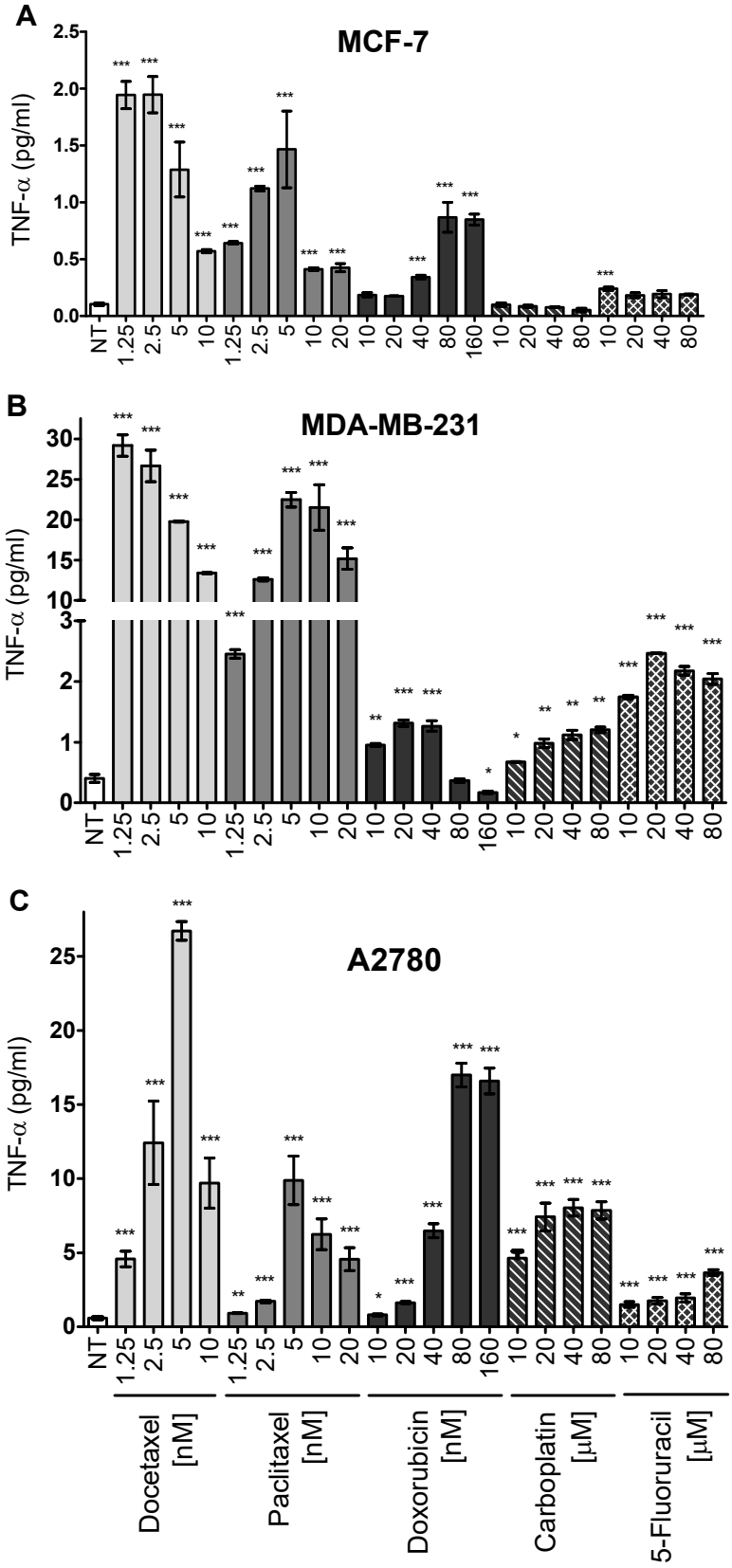
A
MCF-7
TNF-α (pg/ml)
B
MDA-MB-231
TNF-α (pg/ml)
C
A2780
TNF-α (pg/ml)
NT
1.25
2.5
5
10
20
40
80
160
Docetaxel [nM]
Paclitaxel [nM]
Doxorubicin [nM]
Carboplatin [μM]
5-Fluoruracil [μM]

Figure 3.2 - Increased TNF-α release induced in select tumour cell lines by a variety of chemotherapeutic drugs.

Media levels of TNF-α were measured by ELISA after 72 hours of treatment with either docetaxel, paclitaxel, doxorubicin, carboplatin, or 5-FU. Drug concentrations tested were based on IC50 values previously determined experimentally in MCF-7 cells. The data represents the mean of three replicates (+/-SEM). The significance of differences in TNF-α levels between treated and untreated cells was assessed using a two-tailed T-test; *** $p<0.0001$, ** $p<0.001$, * $p<0.05$

3.1.3 Increased levels of TNF-α in the culture medium are due to increased gene transcription, not loss of membrane integrity

In order to provide further insight into the mechanisms by which docetaxel induces the release of TNF-α from tumour cells, we assessed whether docetaxel-induced TNF-α release is merely a passive cellular process resulting from the loss of membrane integrity during cell death, or whether it is an active cellular response to drug exposure, as specified in objective (a-ii). MCF-7 cells were treated for 48 or 72 hours with docetaxel at two different concentrations and the number of trypan blue-positive cells were quantified as a measure of lost plasma membrane integrity (figure 3.3B). All docetaxel treatments caused an increase in the number of trypan blue-positive cells (figure 3.3B). As expected the higher docetaxel concentration (15 nM) resulted in a higher number of trypan blue-positive cells than 2.5 nM docetaxel. In contrast, treatment with 15 nM docetaxel was not associated with the highest concentration of TNF-α in the media (figure 3.3A), suggesting that TNF-α release is not principally associated with drug-induced cytolysis. In order to confirm this interpretation, cells were subjected to hypotonic conditions causing cell-lysis and assessed for their media levels of TNF-α (figure 3.3C). Consistent with the previous interpretation, cell lysis alone did not result in significant increases in secreted TNF-α levels.

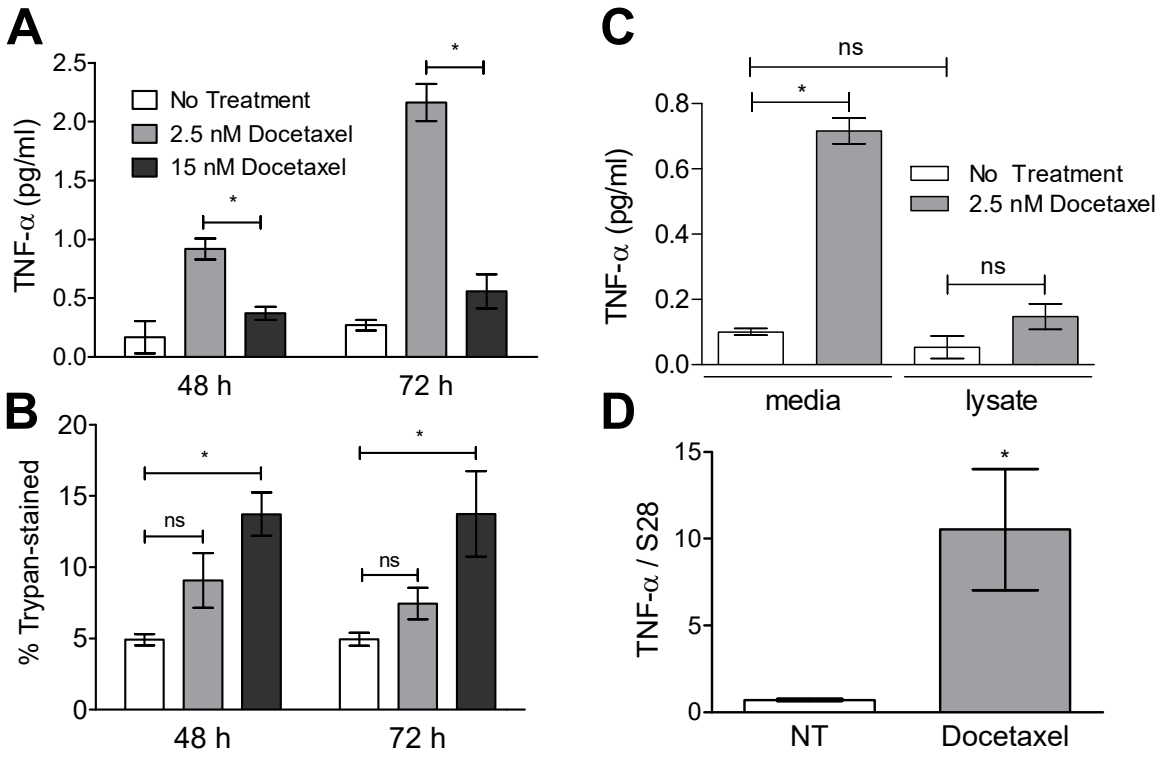

Figure 3.2 - TNF-α release does not correlate with loss of membrane integrity. Cells were treated for 48 or 72 hours with one of two concentrations of docetaxel afterwhich (A) the level of TNF-α in the media was measured as well as (B) the number of trypan-positive cells. The significance of differences in TNF-α levels or loss of membrane integrity were assessed using a 2-way ANOVA with a Tukey post-test. Cells were also lysed under hypotonic conditions and (C) concentrations of TNF-α from lysed and non-lysed cells were measured after 48 hours using an ELISA. (D) TNF-α mRNA levels were also determined by quantitative PCR using cDNA generated from RNA extracts after 36 hours of docetaxel treatment. In these studies, the significance of differences in TNF-α transcript levels between preparations was measured using an unpaired, two-tailed T-test; * $p<0.05$. All data represent the mean of three replicates (+/-SEM).

It has been shown that paclitaxel can induce increased production of TNF-α transcripts in macrophages (133). We thus examined whether the drug-induced increase in media levels of TNF-α in MCF-7 cells involved the ability of docetaxel to induce transcription of the human TNF-α gene. We observed that after 36 hours of exposure to 2.5 nM docetaxel, MCF-7 cells significantly increased their production of TNF-α transcripts by more than ten-fold (figure 3.3D), while at earlier time points no significant increase was observed (see figure A2 in appendix section).

3.2 Comparing cellular responses to docetaxel and lipopolysaccharides in terms of TNF-α production

As reviewed above, it has been suggested that the ability of taxanes to induce the production of inflammatory cytokines involves the *direct* activation of TLR4 (119,200,207,208,210); however, clear evidence for this has yet to be demonstrated in tumour cells. In order to provide further insight into the mechanism by which docetaxel and LPS induce the release of TNF-α from tumour cells, we assessed whether there were differences in the inflammatory cytokine profile that is released when treating the cells with either docetaxel or LPS (as specified in objective (a-iii); data presented in figure 3.4). To address the second part of objective (a-iii), which is to compare/contrast the receptors involved in either docetaxel- or LPS-induced TNF-α release, we inhibited the transmembrane TLR4 receptor at both an intracellular and extracellular domain and looked at the resulting ability of MDA-MB-231 cells to respond with TNF-α release after exposure to either docetaxel or LPS (figure 3.5). LPS and docetaxel reportedly share the ability to stimulate the release of several inflammatory cytokines that have been implicated in resistance to chemotherapy agents, thus we assessed the levels of three of these cytokines, namely TNF-α, CXCL1, and CXCL8.

3.2.1 Cellular response to docetaxel is distinct from that of LPS in breast and ovarian cancer cells

MCF-7 cells responded to LPS with the release of TNF-α, CXCL8, and CXCL1. In contrast, docetaxel only induced detectable increases in TNF-α and CXCL8 expression. A2780 cells, in contrast, responded to docetaxel with the release of TNF-α only, and when challenged with LPS, no significant changes in any of the above cytokines were observed (figure 3.4).

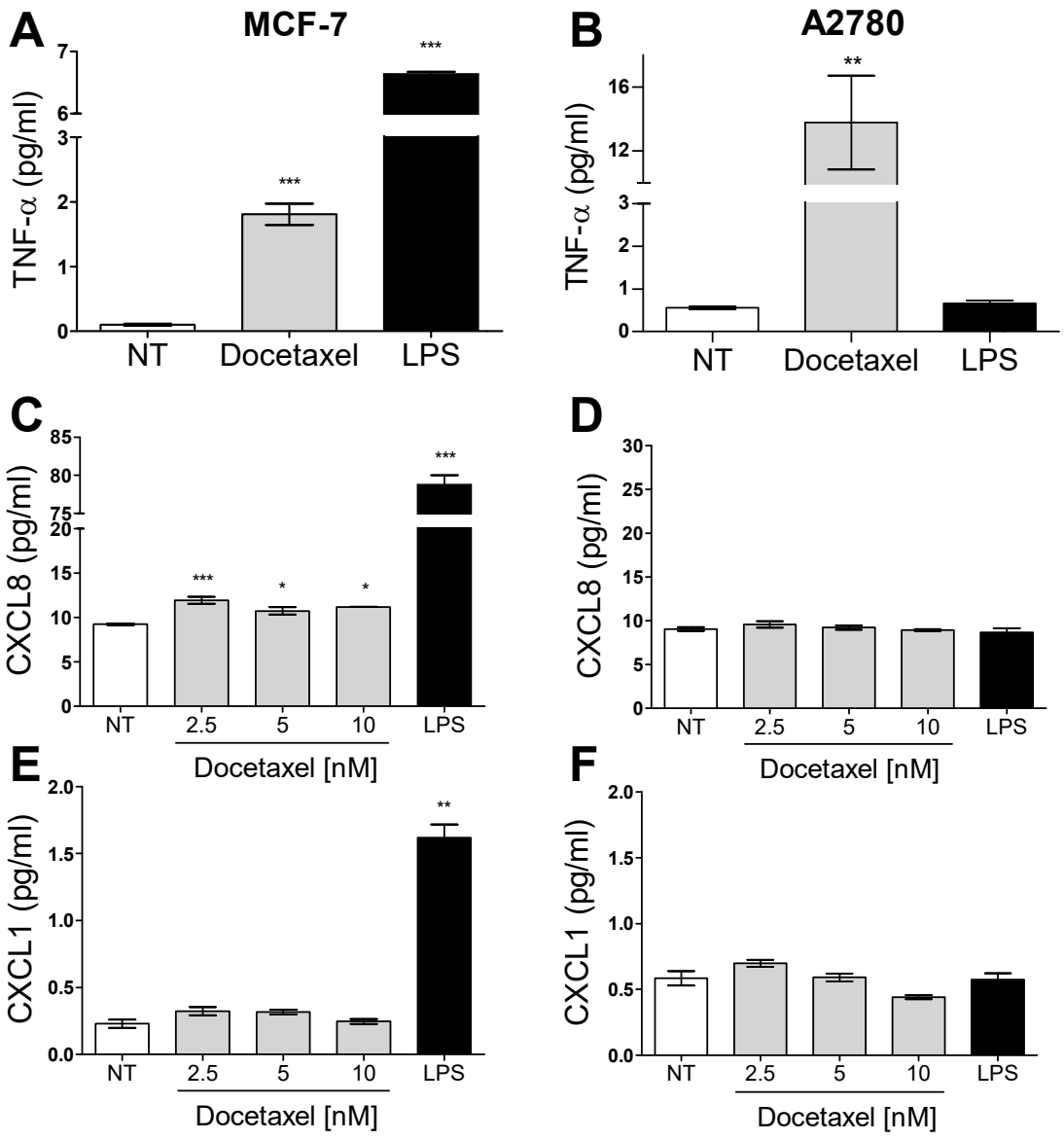

Figure 3.3 - Media cytokine profiles for tumour cells after LPS or docetaxel exposure. The levels of TNF-α (A and B), CXCL8 (C and D), and CXCL1 (E and F) in culture media of either MCF-7 (A, C and E) or A2780 (B, D and F) cells were measured by ELISA after 72 hours of LPS or docetaxel treatment; The data represent the mean of three replicates (+/-SEM). All treatments were of 10 µg/ml for LPS and one of 2.5, 5, or 10 nM for docetaxel; The significance of differences in TNF-α levels between treated and untreated cells were determined using a two-tailed T-test; *** for $p<0.0001$, ** for $p<0.01$, and * for $p<0.01$

Unlike the canonical (hexa-acylated) form of LPS produced in *E. coli*, under-acylated forms such as tetra- and penta-acylated LPS are fundamentally distinct in their interactions with TLR4. Rather they are antagonists of TLR4 activation (212). Penta-acylated LPS is synthesized by some bacteria, including *Rhodobacter Sphaeroides* (213) and elicits an inhibitory effect through the binding of MD-2, forming a complex that suppresses TLR4 activation (212,214,215). Recently, another group has shown that TLR4 activity can also be selectively inhibited by a small molecule called TAK-242 (216). Unlike LPS-RS, TAK-242 associates with the intracellular TIR (toll-interleukin-1 receptor) domain of TLR4 and prevents its association with intracellular adaptor proteins. It was therefore of interest to determine whether inhibition of TLR4 signalling with either penta-acylated LPS from *R. Sphaeroides* (LPS-RS) or TAK-242 would inhibit docetaxel-induced TNF-α production in tumour cells. Using MDA-MB-231 cells (prominent TLR4 expressers) it was found that treatment with an LPS-RS concentration of 100-fold higher than that of LPS caused complete inhibition of LPS-induced TNF-α release, consistent with experiments in human monocytes (213). However, LPS-RS did not suppress docetaxel's ability to induce TNF-α release, but rather augmented it (figure 3.5A). In a similar experiment the LPS antagonist TAK-242 was administered in combination with either docetaxel or LPS (figure 3.5B). As with LPS-RS, TAK-242 completely abrogated LPS-induced TNF-α release from MDA-MB-231 cells, however did not significantly affect docetaxel-induced TNF-α release. Together these findings demonstrate that interference with the LPS-binding domain (figure 3.5A) or blocking the association between the intracellular TIR (toll-interleukin-1 receptor) domain of TLR4 and

its adaptor molecules did not prevent docetaxel-induced TNF-α release, while abrogating that induced by LPS.

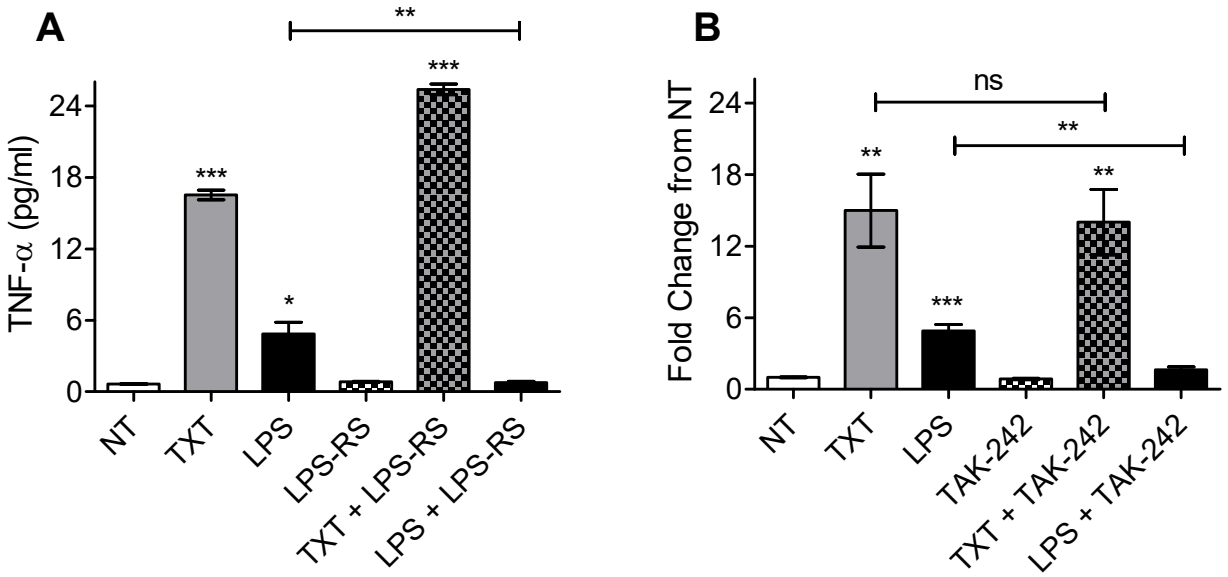

Figure 3.4 - The effect of TLR4 inhibition on the release of TNF-α induced by docetaxel or LPS. MDA-MB-231 cells were treated for 72 hours with either 2.5 nM docetaxel (TXT) or 0.1 µg/ml LPS, after pre-treatment with either 100 µg/ml LPS-RS (A) or 0.1 µg/ml TAK-242 (B). The significance of differences between treated and untreated cells was assessed using a two-tailed T-test; ***p<0.001, **p<0.01, *p<0.02

3.2.2 Inhibition of docetaxel-induced TNF-α release by Marimastat

In macrophages it is thought that TNF-α release, particularly in response to LPS, occurs by constitutive exocytosis, involving a receptor-mediated increase in gene transcription (217,218). Increased gene transcription results in increased production of a membrane-bound precursor of TNF-α (mTNF-α), and TNF-α release involves cleavage of mTNF-α to produce the soluble form (sTNF-α) (192). In order to further address objective (a-iii), we inhibited a key element in the cleavage of mTNF-α, which is part of the family of matrix metalloproteinases, called TNF-α converting enzyme (TACE), also known as ADAM-17, and assessed the effect on docetaxel-induced TNF-α release. Hydroxamate-based agents cause inhibition of a broad-spectrum of matrix metalloproteinases (MMPs) (219), and have also been shown to inhibit TNF-α release, suggesting that one or more MMPs are involved in this process, including TACE (192). Although the pathways governing cytokine trafficking and release have been well-studied in macrophages and in other murine cell lines, there is a lack of understanding about the pathways responsible for cytokine trafficking in epithelial cells (217) or in epithelial-derived tumour cells. It was therefore of interest to determine whether similar mechanisms are employed during the processing of drug-induced TNF-α release in epithelial-derived tumour cell lines. We thus examined the effect of the hydroxamate-based MMP inhibitor Marimastat on LPS- and docetaxel-induced TNF-α release from various cell lines. Marimastat reportedly

inhibits MMP-1, 2, 3, 7, 8, 9, and 14, as well as ADAM-17 with respective IC_{50} values of 5, 6, 200, 20, 2, 3, 1.8, 3.8 nM (219).

Marimastat did not have a significant effect on docetaxel-induced TNF-α release from MCF-7 cells (figure 3.6A). In contrast, LPS-induced TNF-α release was significantly diminished (by ~50%) in the presence of Marimastat. Our findings suggest that in MCF-7 cells, LPS-induced TNF-α release is mediated, at least in part, by shedding of TNF-α from the plasma membrane by MMPs. It was, however, unclear whether MMPs play a role in docetaxel-induced TNF-α release from MCF-7. Supporting the role of MMPs in TNF-α release, we observed that Marimastat inhibits basal and docetaxel-induced TNF-α release from A2780 cells (figure 3.6B). The differential sensitivity to Marimastat suggests that the mechanisms for docetaxel-induced TNF-α release in MCF-7 and A2780 cells are distinct.

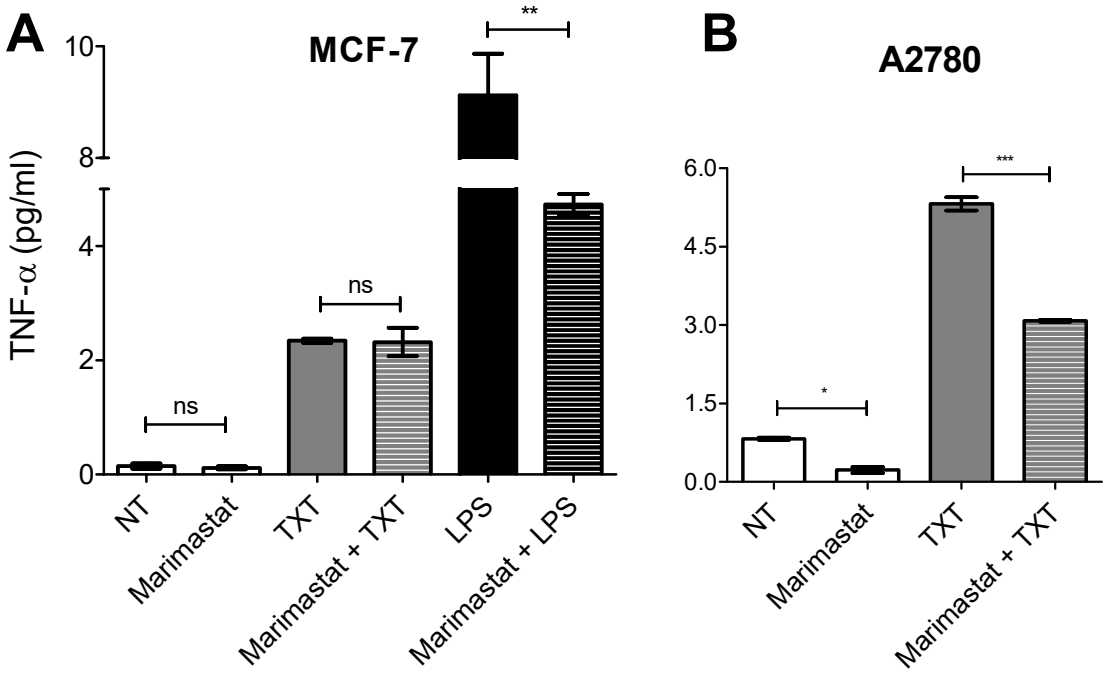

Figure 3.6 - The effect of MMP inhibition by Marimastat on the release of TNF-α. MCF-7 (A) and A2780 (B) cells were treated with 2.5 nM docetaxel (TXT) or 10 µg/ml LPS for 72 hours in the presence or absence of 200 nM Marimastat, a broad-spectrum MMP inhibitor. The significance of differences in TNF-α levels between treatments was assessed using a two-tailed T-test; *** $p<0.0001$, ** $p<0.001$, *$p<0.01$

3.3 Changes in TNF-α production upon selection for docetaxel resistance

3.3.1 P-glycoprotein (Abcb1) mediates the docetaxel-resistant phenotype

A series of docetaxel-resistant breast tumour cell lines (MCF-7_{TXT7} to MCF-7_{TXT12} cells) were previously created by selection of wild-type MCF-7 cells for survival in increasing concentrations of docetaxel (28). In order to characterize the drug-resistant phenotype exhibited in our cell lines, as specified in objective (c-i), we assessed the above series of docetaxel-resistant tumour cell lines in terms of their level of P-gp expression at the protein level and their ability to prevent the accumulation of docetaxel (figure 3.7). This ability of tumour cells is often found to be a primary mode of chemotherapy drug resistance and our findings suggest that the primary mode of drug resistance in our drug-resistant cells was attributable to P-gp-mediated drug extrusion (figure 3.8).

These tumour cell lines were shown to exhibit progressively increased transcription of the gene encoding P-glycoprotein (P-gp, also known as Abcb1) relative to cells that were grown in the absence of drug (220). To confirm that this progressive increase in gene transcription corresponded to increases in P-gp protein levels, we conducted P-gp-antibody-based experimentation to quantify the relative amount of protein for each of the drug-selected MCF-7 tumour cell lines.

As shown in figure 3.7A, MCF-7_{TXT} cells exhibited progressively increased P-gp protein expression (relative to the MCF-7_{CC10} cell line), which correlated with their observed level of docetaxel resistance. Detectable increases in P-gp protein expression (relative to MCF-7 co-cultured control cells) first occurred at selection dose 9 (MCF-7_{TXT9} cells), which is also the selection dose at which docetaxel resistance is first achieved. Consistent with P-gp's ability to transport docetaxel (221) and other chemotherapy drugs out of tumour cells (222), we observed that docetaxel-resistant MCF-7 cells showed decreased uptake of tritium-labeled docetaxel (H^3-TXT), relative to the drug-sensitive MCF-7_{CC10} cell line (figure 3.7B). In order to assess whether P-gp was responsible for the decreased docetaxel uptake, a specific allosteric inhibitor for P-gp, Tariquidar, was given in combination with docetaxel (figure 3.7B). It was found in MCF-7_{TXT10} cells that drug uptake was restored significantly upon addition of Tariquidar, thus confirming the role of P-gp in the export of docetaxel from drug-selected cells.

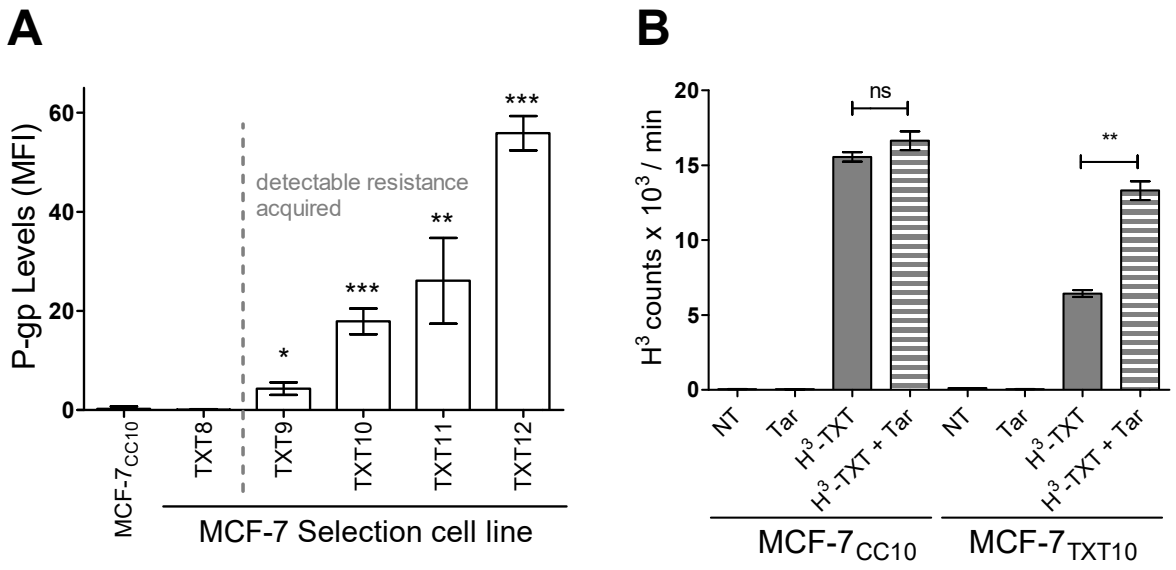

Figure 3.7 - Increased P-gp levels are associated with diminished drug uptake in drug-resistant MCF-7 cells. (A) Flow cytometry was used to assess P-gp levels (as expressed by mean fluorescence intensity (MFI) values) for MCF-7 cells at selection doses 8, 9, 10, 11, and 12. (B) Docetaxel-resistant MCF-7_{TXT10} cells were assessed for their intracellular drug accumulation with or without the addition of Tariquidar (Tar), an allosteric inhibitor of P-gp. Tritiated docetaxel (H^3-TXT) was administered at a concentration of 2.5 nM, with or without 100 nM Tariquidar treatment for 12 hours and H^3-TXT accumulation was then determined by measuring the radioactivity of cells. All data represents the mean of 3 replicates (+/-SEM). Two-tailed T-tests were employed to assess the significance of differences in cellular H^3-TXT accumulation between the various treatments in the control (MCF-7_{CC10}) and docetaxel-resistant (MCF-7_{TXT10}) cell lines; *** for $p<0.0001$, **$p<0.001$, and * for $p<0.01$

Given that increased P-gp levels were shown to affect docetaxel uptake in docetaxel-resistant cells, we assessed whether restoration of drug-uptake would also restore docetaxel sensitivity. As shown in figures 3.8A and B, pre-incubation of docetaxel-resistant MCF-7_{TXT10} (IC_{50} = 7.14 nM without Tariquidar) with the P-gp inhibitor Tariquidar increased sensitivity to

docetaxel (IC_{50} = 0.42 nM with Tariquidar). Likewise, pre-incubation of docetaxel-resistant $A2780_{DXL12}$ cells (IC_{50} = 47.5 nM without Tariquidar) with the P-gp inhibitor Tariquidar also increased sensitivity to docetaxel (IC_{50} = 0.435 nM with Tariquidar). On the otherhand, pre-incubation of the co-culture control drug-sensitive MCF-7_{CC10} (IC_{50} = 0.24 nM without Tariquidar) and $A2780_{CC12}$ (IC_{50} = 0.630 nM without Tariquidar) did not have a significant effect on drug sensitivity to docetaxel (IC_{50} = 0.28 nM and 0.331 nM with Tariquidar for MCF-7_{CC10} and $A2780_{CC12}$ cells, respectively). The ability of Tariquidar to restore drug sensitivity in drug-resistant cells to a level that is close to their respective co-cultured control cells suggests that the docetaxel-resistant phenotype is primarily achieved by P-gp–mediated drug efflux from the cells.

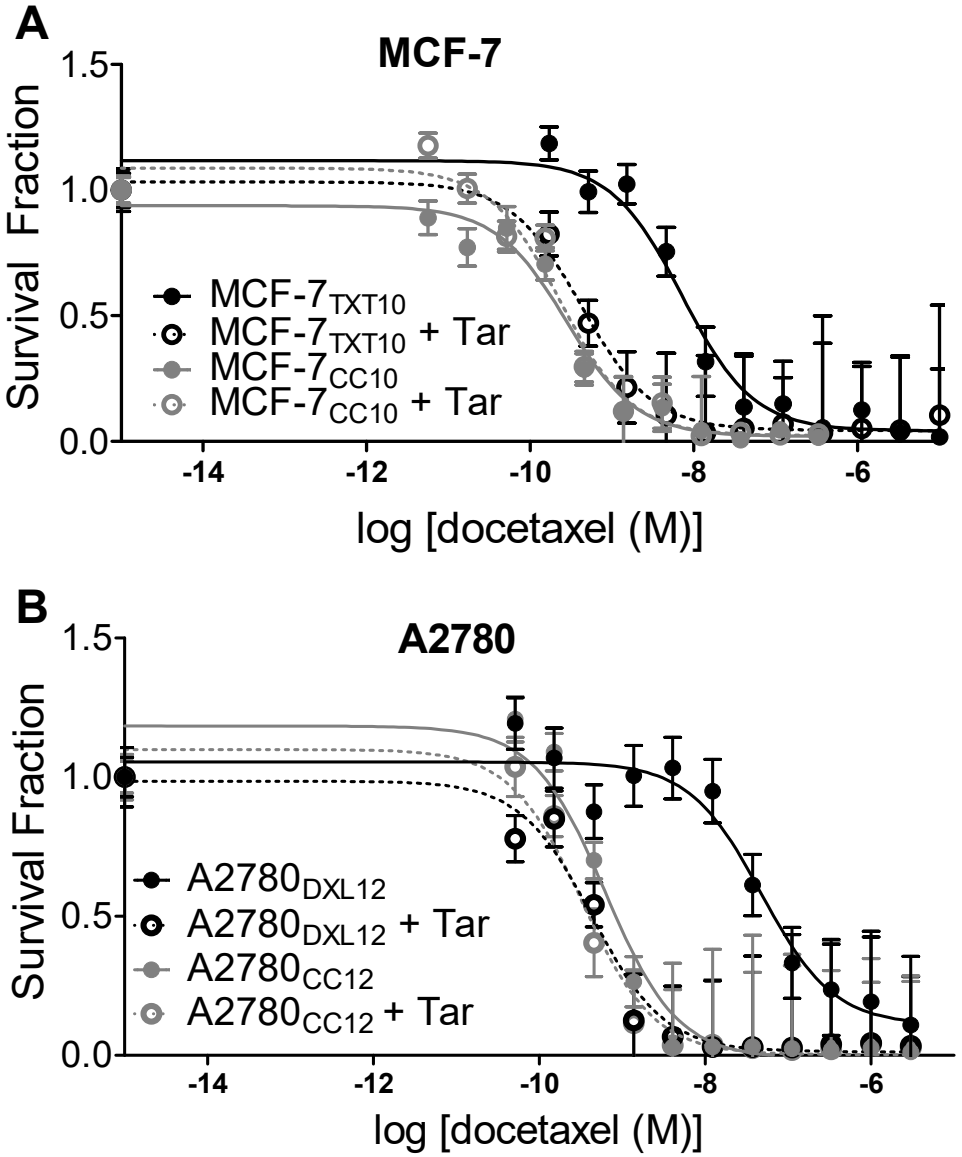

Figure 3.8 - Sensitivity to docetaxel is restored upon inhibition of P-gp activity with Tariquidar (Tar). (A) Clonogenic assays yielded IC_{50} values for docetaxel in MCF-7_{CC10} cells of 0.28 nM and 0.24 nM, with and without 100 nM Tariquidar, respectively. IC_{50} values for MCF-7_{TXT10} cells were significantly different with (0.42 nM) and without (7.14 nM) Tariquidar, respectively ($p<0.0001$). (B) An identical trend was observed for the $A2780_{CC12}$ (IC_{50} = 0.630 nM) and $A2780_{DXL12}$ (IC_{50} = 47.5 nM) cell lines, where in the presence of Tariquidar IC_{50} values were 0.331 nM and 0.435 nM, respectively. Each data point represents the mean number of colonies in twelve independent

microscopic fields divided by the mean number of colonies in the untreated control (+/-SEM). Each experiment was replicated three times with consistent trends. Non-linear regression analysis was used to compare the significance of difference in IC_{50} values between Tariquidar-treated and untreated conditions.

3.3.2 Basal production of multiple cytokines increases in tumour cell lines selected for resistance to docetaxel

In order to fulfill objective (c-ii), the release of three inflammatory cytokines, known to be contributors in the tumour drug-resistant phenotype, was measured from cell lines selected for survival in increasing concentrations of docetaxel (figure 3.9).

Previous reports by our laboratory have shown that MCF-7 cells selected for resistance to docetaxel exhibited increased basal levels of TNF-α. At very high selection doses, TNF-α production eventually returned to the level of co-cultured control cells (24). In the same study, it was reported that acquisition of docetaxel resistance in MCF-7 cells was associated with decreased expression of TNF-α receptor 1 protein (TNFR1) relative to drug-sensitive control cells, thereby diminishing TNF-α's ability to induce apoptosis. The increased TNF-α production was thought to contribute to the resistant phenotype by activating TNFR2-dependent survival pathways, although TNFR2 blockade accounted for a relatively small restoration of drug sensitivity relative to that achieved by inhibiting NF-κB (24) or P-gp activity (figure 8). Activation and subsequent nuclear localization of NF-κB can occur in response to a variety of extracellular stimuli, including endogenously produced inflammatory cytokines, such as TNF-α and CXCL8 (223) as well as CXCL1 (224), thus we assessed whether increases in TNF-α production, as well

as CXCL8 and CXCL1, would also be observed during selection of tumour cells for increasing resistance to docetaxel.

As shown in figure 3.9, basal TNF-α levels increased upon selection for docetaxel resistance, when the selection dose reached level 9 and 10 (MCF-7_{TXT9} and MCF-7_{TXT10} cells), beyond which selection at higher doses resulted in TNF-α levels subsiding back to that of non-selected cells. Similarly, it was also found that basal levels of CXCL8 and CXCL1 increased with maximum production in MCF-7_{TXT10} cells, beyond which their levels also declined toward those of non-selected cells (figure 3.9). Interestingly, $A2780_{DXL}$ cells above selection dose 10 showed a similar elevation in basal TNF-α and CXCL1 levels (figure 3.9B and F); however, no changes in CXCL8 expression were observed (figure 3.9D).

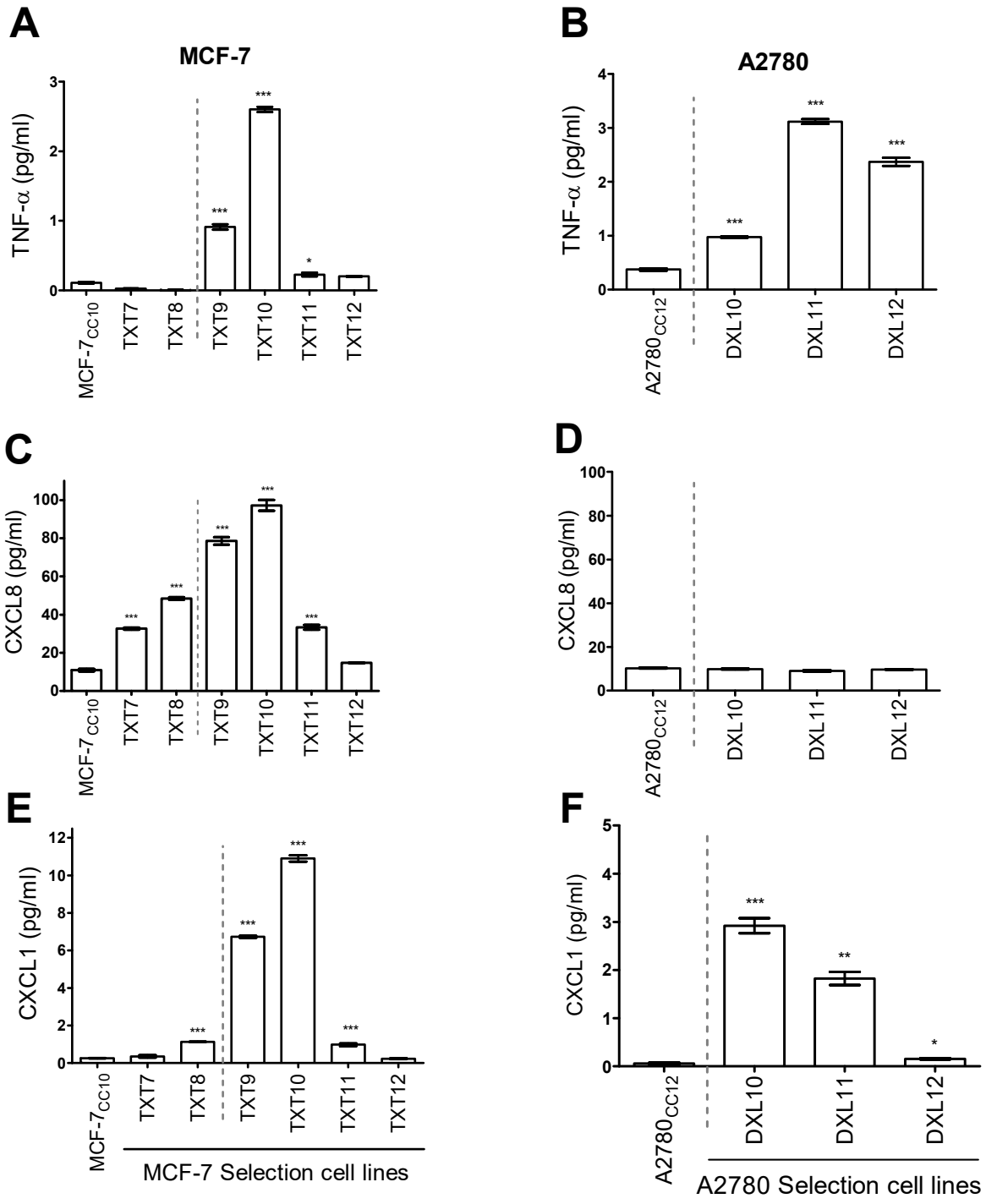
A
MCF-7
TNF-α (pg/ml)
MCF-7CC10
TXT7
TXT8
TXT9
TXT10
TXT11
TXT12
B
A2780
TNF-α (pg/ml)
A2780CC12
DXL10
DXL11
DXL12
C
CXCL8 (pg/ml)
D
CXCL8 (pg/ml)
E
CXCL1 (pg/ml)
MCF-7 Selection cell lines
F
CXCL1 (pg/ml)
A2780 Selection cell lines

Figure 3.9 - Basal cytokine production changes during selection for resistance to docetaxel. Levels of TNF-α (A and B), CXCL8 (C and D), and CXCL1 (E and F) in media as measured by ELISA, representing media levels of cytokines in MCF-7 (A, C, and E) and A2780 cells (B, D, and F) after 72 hours of cell culture. Cell lines to the right of the vertical broken grey line exhibit acquired resistance to docetaxel, as confirmed in clonogenic assays. Each value represents the mean of three replicates (+/-SEM). Two-tailed T-tests were used to assess the significance of differences in cytokine levels between docetaxel-selected cell lines and their respective co-cultured control cell lines (MCF-7_{CC10} or $A2780_{CC12}$); ***p<0.0001, **p<0.001, *p<0.01

3.3.3 Elevated basal production of TNF-α in docetaxel-resistant cells is inhibited upon treatment with a broad-spectrum MMP Inhibitor

In an effort to compare the TNF-α release pathway(s) employed by drug-resistant cells to that of drug-naive cells, we conducted similar experimentation, as presented in figure 3.6, to assess the effect of MMP inhibition on the level of basal release of TNF-α in drug-resistant breast tumour cells. As mentioned, TNF-α is first synthesized as an integral membrane protein that is typically released from cells through the action of the metalloproteinase ADAM-17 (192). We thus examined the effect of the MMP inhibitor Marimastat on the ability of docetaxel-resistant MCF-7 cells to produce elevated basal levels of TNF-α. As shown in figure 3.10, Marimastat was able to inhibit basal TNF-α production in MCF-7_{TXT10} cells, suggesting that this process depends, at least in part, on MMP activity and shedding of membrane-bound TNF-α. Unlike the previous observations in the drug-naive MCF-7 cell line, the production of TNF-α in MCF-7_{TXT10} cells was not augmented by treatment with 2.5 nM docetaxel for 72 hours. In contrast to docetaxel, LPS was able to strongly augment TNF-α production, and this production was also inhibited by Marimastat. TNF-α release was not fully abolished by Marimastat under any conditions.

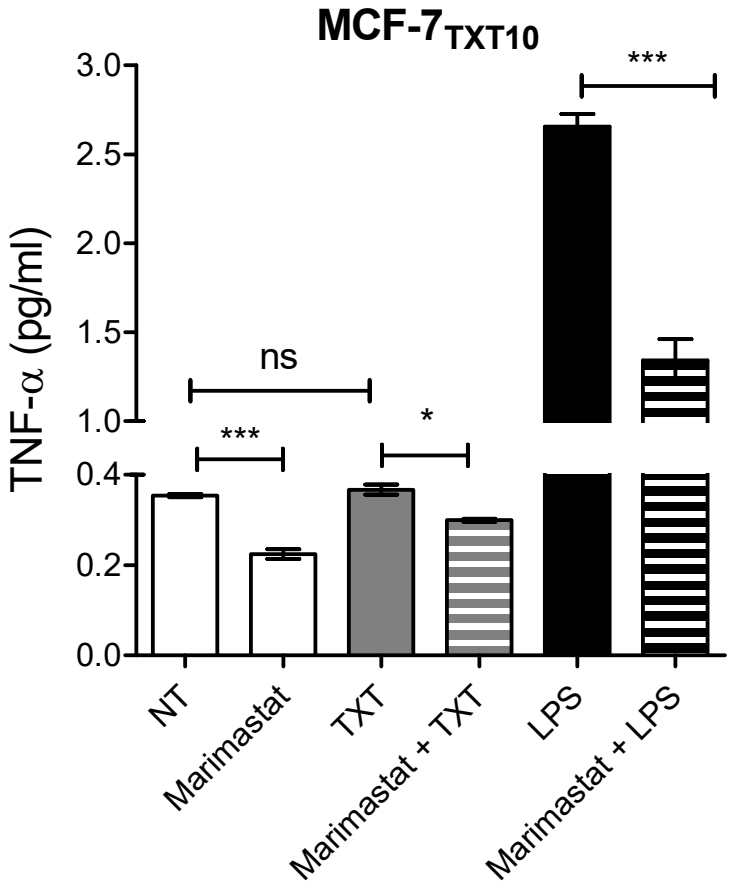

Figure 3.10 - Effect of Marimastat on TNF-α levels in the medium of MCF-7_{TXT10} cells. Cells were treated for 72 hours with media only (NT), 200 nM Marimastat, 2.5 nM docetaxel (TXT), 10 μg/ml LPS, or a combination. The data represents the mean of three replicates (+/-SEM) and the significance of differences in TNF-α levels between treatments was determined using a T-test; *p<0.01, **p<0.001, ***p<0.0001

3.3.4 Differences in TNF-α release pathways in tumour cells

Docetaxel-resistant MCF-7_{TXT10} and $A2780_{DXL12}$ cells failed to increase their output of TNF-α in response to a 72-hour docetaxel exposure, in contrast to their respective co-cultured control cell lines. This was in contrast to the effect observed using LPS, which resulted in significant increases in TNF-α release in both docetaxel-sensitive (MCF-7_{CC10}) and docetaxel-resistant (MCF-7_{TXT10}) cells (figure 3.11A). In contrast, docetaxel-resistant $A2780_{DXL12}$ cells remained unresponsive to LPS, along with respective co-cultured control cells (figure 3.11B), as previously described (figure 3.4). In an effort to identity potential receptors and pathway components involved in TNF-α release from tumour cells, in support of objective (a-iii), immunoblot experiments were conducted to measure TLR4 and MyD88 (a TLR4 adaptor protein) expression at the protein level for both MCF-7 as well as A2780 cells. Immunoblots revealed that both MCF-7 and A2780 cell lines expressed the cell-surface receptor TLR4 (figure 3.11C and D). MyD88 (a TLR4 adaptor protein) levels were detectable in MCF-7 cells and were elevated greater than three-fold in docetaxel-resistant cells (MCF-7_{TXT}) (figure 3.11C). By contrast, MyD88 was undetectable in both A2780 and their docetaxel-resistant counterparts ($A2780_{DXL}$) (figure 3.11D), identifying MyD88 expression as a potential requirement for LPS-induced TNF-α release but not for that induced by docetaxel.

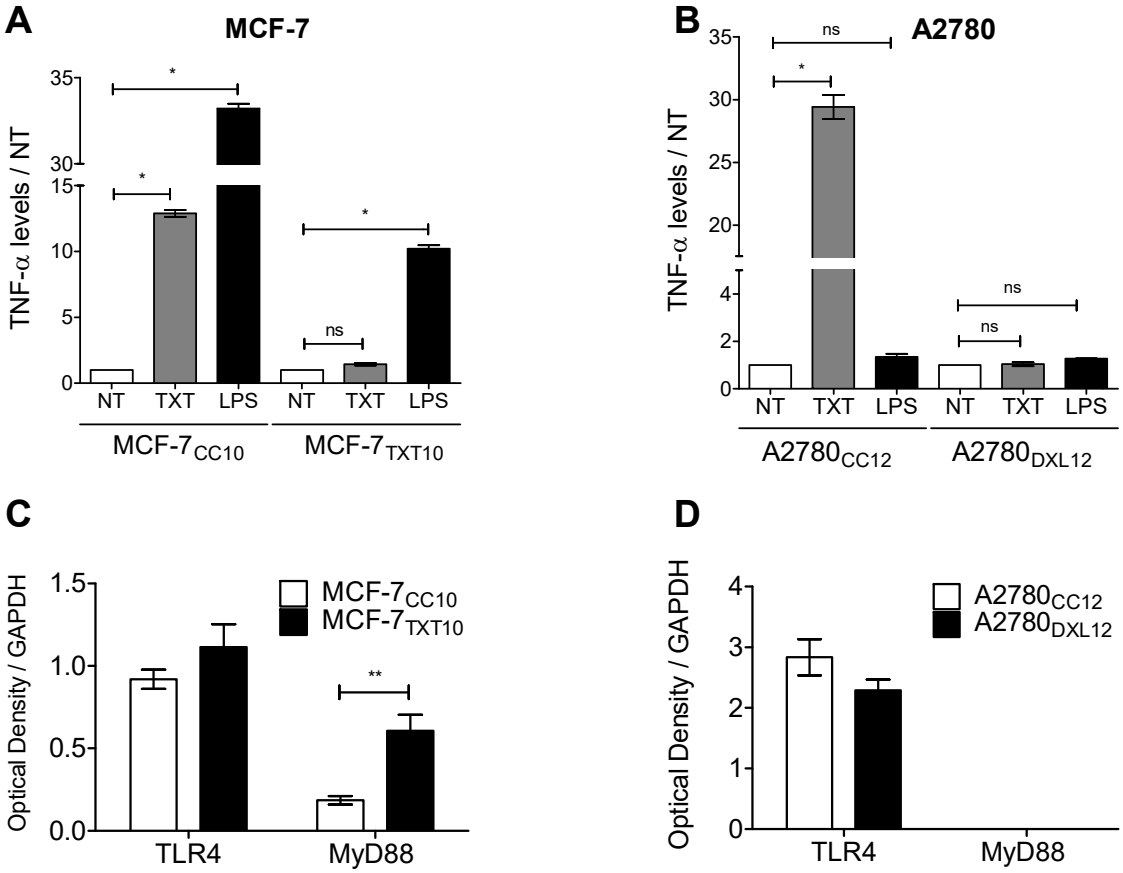

Figure 3.11 - Comparing the effects of docetaxel and LPS treatment on TNF-α release from drug-naive and drug-resistant cell lines and assessment of TLR4/MyD88 levels. (A and B) Docetaxel (TXT) concentrations used were 2.5 nM and LPS concentrations were 5 µg/ml. Significance of differences in TNF-α release between treated and untreated cells were determined using a one-way ANOVA with a Tukey post-test; * for $p<0.05$; all data points represent the mean of three trials. (C and D) Levels of TLR4 and MyD88 were assessed for each cell line and statistical analysis comparing drug-naive and drug-resistant cells consisted of a two-tailed T-test; ** for $p<0.01$.

S

3.3.5 Restoring cellular drug accumulation potentiates drug-induced TNF-α release in docetaxel-resistant cells

The fact that docetaxel-induced TNF-α release was found to diminish after MCF-7 cells were selected for survival in increasing concentrations of docetaxel, prompted us to assess whether the ability of drug-resistant cells to extrude docetaxel was a contributing factor, allowing us to further address objective (a-iii). Given the presence of P-gp and its contribution to reduced cellular docetaxel accumulation in docetaxel-resistant, MCF-7_{TXT} and $A2780_{DXL}$, cells (figure 3.7), we examined the effect of the P-gp inhibitor Tariquidar on docetaxel-induced TNF-α production in these cell lines. As shown in figure 3.12, pre-incubation with Tariquidar had no significant effect on drug-induced TNF-α release in co-cultured control MCF-7 and A2780 cells. In contrast, Tariquidar potentiated docetaxel-induced TNF-α release from MCF-7_{TXT} and $A2780_{DXL}$ cells, suggesting that docetaxel accumulation within tumour cells (drug entry) is required for drug-induced TNF-α release.

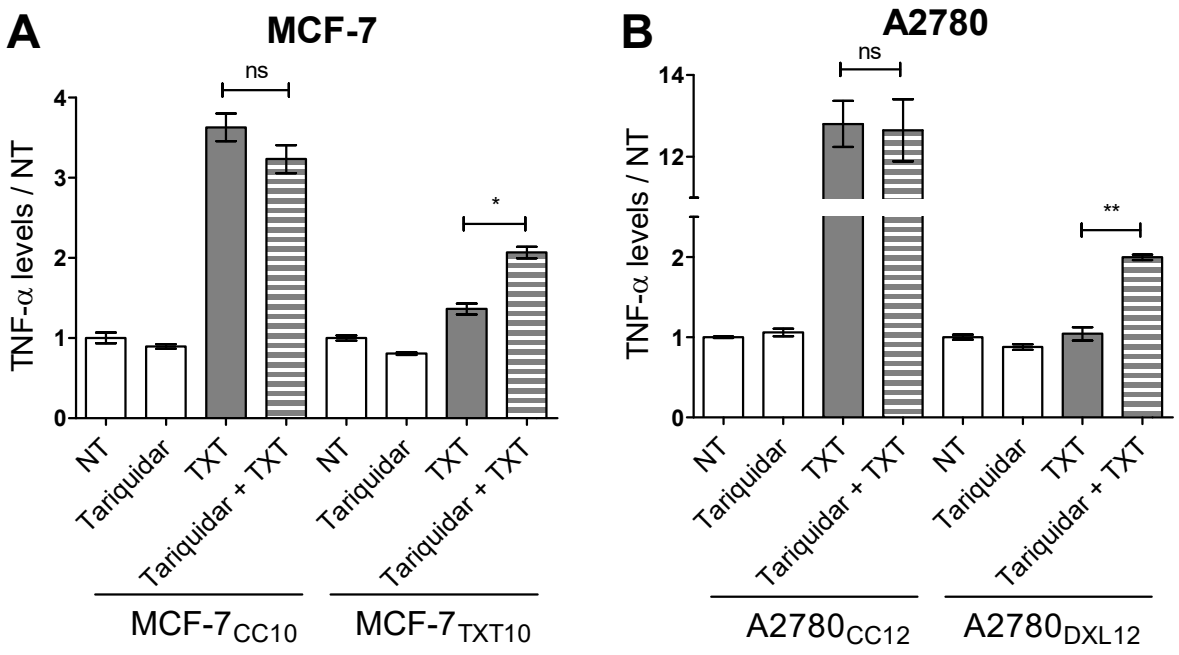

Figure 3.12 - Effects of Tariquidar on docetaxel-induced TNF-α release. All treatments lasted 72 hours at which point levels of TNF-α in the media were measured by ELISA for (A) MCF-7 and (B) A2780 cells. Cells were either untreated or treated with 100 nM Tariquidar and/or 2.5 nM docetaxel (TXT). The data are representative of three replicates (+/-SEM). A two-tailed T-test was used to assess the significance of differences in cellular TNF-α release between treatments; ** for $p<0.001$, * for $p<0.01$

3.4 Effects of LPS pre-treatment on docetaxel cytotoxicity

Given our data showing that LPS induces increased TNF-α production in drug-resistant tumour cells (MCF-7_{TXT10}) where docetaxel could not, it was hypothesized that LPS might augment docetaxel cytotoxicity if administered in combination with docetaxel, as stated in hypothesis (c). In order to test this hypothesis, a series of drug sensitivity assays were conducted. In contradiction with this hypothesis, however, TLR4 pathway stimulation has been demonstrated to result in *decreased* taxane cytotoxicity, specifically in PC-3 prostate cancer cells (225) and in MyD88-positive (SKOV3 and OVCAR-3) ovarian cancer cells, but to have no effect in MyD88-negative (A2780 and 3AO) ovarian cancer cells (209). Therefore it was of interest to assess the potential effects of LPS in modulating docetaxel cytotoxicity, for MyD88-positive (MCF-7 and MCF-7_{TXT10} cells) and MyD88-negative (A2780 and $A2780_{DXL12}$ cells) tumour cell lines.

3.4.1 LPS induces a docetaxel-sensitizing effect only in tumour cells that exhibit LPS-induced TNF-α release

Our results demonstrate that LPS pretreatment increased docetaxel cytotoxicity in MCF-7_{CC10}, where IC_{50} values were significantly diminished from 0.4 nM (without LPS) to 0.09 nM docetaxel (with LPS). Likewise, MCF-7_{TXT10} cells also experienced a significant increase in

sensitization to drug when pretreated with LPS, given that their IC_{50} values dropped from 27.6 nM (without LPS) to 6.4 nM docetaxel (with LPS; see figure 3.13A), with both cell lines experiencing roughly a 4 to 5-fold increase in drug sensitivity after pretreatment with LPS. In contrast, the sensitivity of $A2780_{CC12}$ cells was unchanged upon addition of LPS (IC_{50} = 1.0 nM with and without LPS). Similarly, the sensitivity of $A2780_{DXL12}$ cells to docetaxel was not significantly affected by pretreatment with LPS (IC_{50} = 28.7 nM without LPS and 21.0 nM with LPS; see figure 3.13B) consistent with their inability to manifest TNF-α release in response to LPS (figure 3.11B).

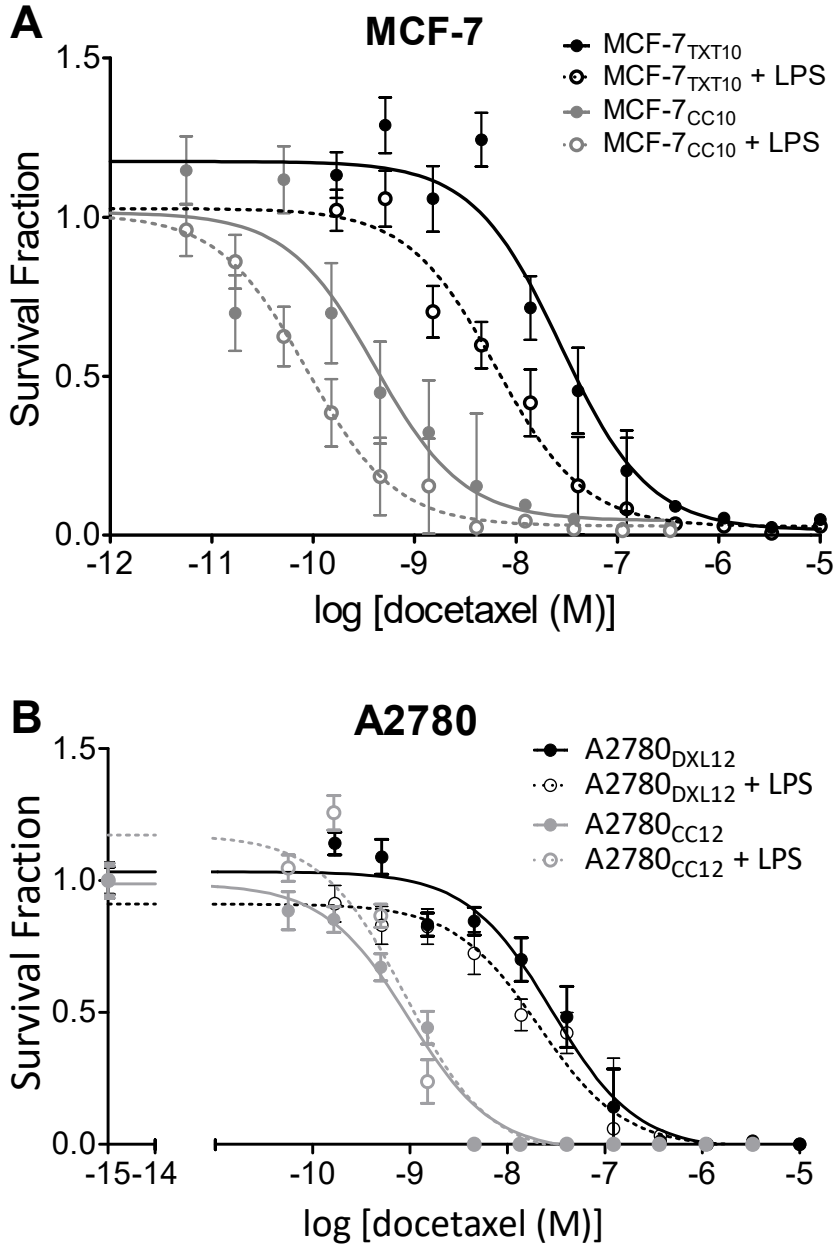

Figure 3.13 - Effect of LPS exposure on tumour cell sensitivity to docetaxel. (A) Sensitivity to docetaxel was assessed using the clonogenic assay. MCF-7_{CC10} cells exhibited an IC_{50} value for docetaxel of 0.4 nM docetaxel, and when pretreated with 10 µg/ml LPS, the value was reduced to 0.09 nM docetaxel. MCF-7_{TXT10} cells exhibited an IC_{50}

value for docetaxel of 27.6 nM docetaxel, which was reduced to 6.4 nM upon addition of 10 µg/ml LPS. Non-linear regression analysis comparing curves generated from LPS-treated and LPS-absent preparations revealed statistically significant differences in IC_{50} values ($p<0.005$). (B) LPS-treated and LPS-absent $A2780_{CC12}$ cells both exhibited an IC_{50} value of 1.0 nM docetaxel and likewise there was no significant difference in the IC_{50} values for docetaxel between LPS-treated and LPS-absent $A2780_{DXL12}$ cells (28.7 nM and 21.0 nM, respectively). Each data point represents the mean of 12 microscopic field counts divided by the mean of the untreated control and each curve is representative of three replicate experiments, each showing a consistent trend.

3.4.2 Docetaxel-sensitizing effect of LPS does not involve changes in cellular drug accumulation

Given the observed ability of LPS to sensitize MCF-7 cells to docetaxel, it was of interest to determine whether this effect involved an increase in the accumulation of drug within the cells. It was found that drug-resistant as well as drug-naive MCF-7 cells pretreated with LPS did not significantly increase their accumulation of radiolabeled docetaxel compared to cells pretreated with only media. This suggests that the drug-sensitizing effect of LPS is attributable to a mechanism other than increased drug accumulation and thus not likely to involve any modulations in P-gp activity.

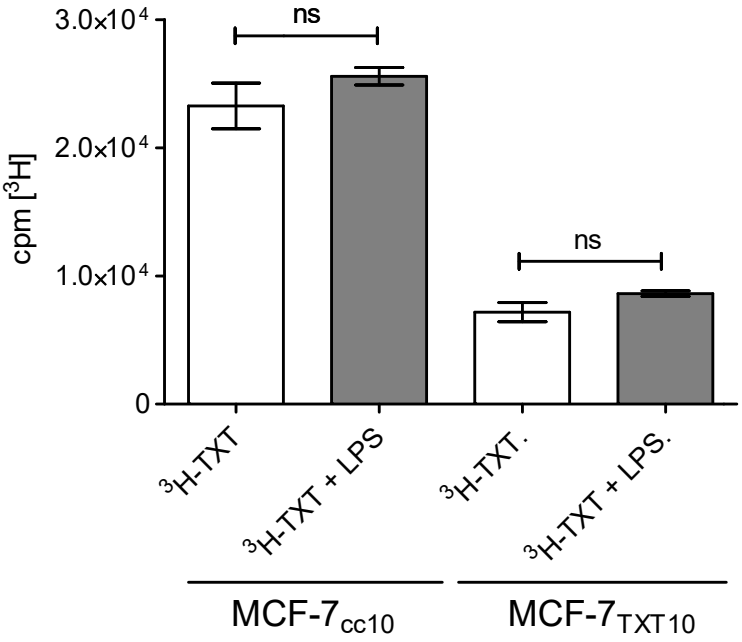

Figure 3.14 - Effects of LPS pretreatment on cellular drug accumulation (uptake). Cells in culture were treated for 48 hours with either 10 µg/ml LPS or media only afterwhich all cell preparations were given 2.5 nM of H^3-TXT for 12 hours and radioactivity of the adhered cell fraction was assessed for each. The data are representative of three replicates (+/-SEM). A one-tailed T-test was used to assess the significance of differences in radioactivity (as measured by counts per minute (cpm)) between LPS-pretreated and non-pretreated cells.

Chapter 4 - Discussion

4.1 Characterization of docetaxel-induced TNF-α release

Findings in this study suggest that a variety of human tumour cell lines respond to docetaxel by increasing their release of TNF-α, similar to the reports of TNF-α release in murine macrophages (133). However, it should be noted that the kinetics of TNF-α release are remarkably distinct in our study. For example, reports of taxane-induced TNF-α production in macrophages involve concentrations of paclitaxel in the 1 to 10 μM range, producing a maximal response within 90 minutes (133). In contrast, tumour cells in this study showed a much more delayed response to taxane treatment, with maximal release of soluble TNF-α at 96 hours, among the treatment times tested (figure 3.1A and B). The response was also maximal at much lower concentrations of docetaxel (between 1.25and 5 nM) (figure 3.1C and D) than reported in macrophages. These contrasting observations suggest a difference in the mechanism of TNF-α release between human tumour cells and murine macrophages. Although much higher concentrations of paclitaxel were used in the previously conducted study, it is possible that exposure of murine macrophages to lower drug concentrations over longer time intervals might also result in TNF-α release. From a clinical perspective, peak plasma concentrations for docetaxel in cancer patients can be as high as 5 μM (226), however it is likely that the concentration of drug that reaches a solid tumour would be significantly lower than that found in the plasma. Studies have shown that doxorubicin concentrations decrease in a solid tumour

as the distance from the nearest blood vessel increases (80), though it is unclear whether docetaxel distributes similarly within tumour tissue. We also observed that a variety of structurally distinct chemotherapy drugs with distinct mechanisms of action are able to induce TNF-α release from breast and ovarian tumour cell lines (figure 3.2). Among the drugs and respective concentrations tested, the taxanes most effectively and consistently induced TNF-α release. However, it is not clear whether the optimal concentration for TNF-α release was identified for each drug tested, and whether the optimal concentrations vary between cell lines. Our study concludes that despite their activation of distinct biochemical pathways and modes of cytotoxicity, the chemotherapy agents tested each have the capacity to activate the TNF-α release pathway(s) with similar kinetics in a variety of human tumour cell lines. With respect to hypothesis (a), data from figure 3.1, 3.2 and 3.3A, proves that drug-induced TNF-α release is dose- and time- dependent and can occur in response to acute exposure to a variety of chemotherapy agents in tumour cells originating from both the breast and the ovarian epithelium.

While the release of TNF-α from cells after exposure to any cytotoxic agent could be the result of cytolysis or secondary necrosis, our findings suggest that docetaxel-induced TNF-α release is not a passive process resulting from cell lysis. Rather, the elevated TNF-α release upon docetaxel treatment appears to involve the increased transcription of TNF-α mRNA, based on quantitative reverse-transcription PCR (figure 3.3). The fact that increases in TNF-α mRNA only occur after a relatively long exposure time to drug (36 hours; See appended figure A2 for

preliminary data showing time course from 18 to 72 hours) suggests that this may be an indirect effect involving one or more intermediary products, including changes in gene expression. At this time, it is unclear as to what these intermediary products might be. The ability of MCF-7 tumour cells to respond to taxane treatment with increased TNF-α gene transcription is shared by murine macrophages, albeit in response to a different taxane, paclitaxel, and with maximal response occurring after 90 minutes of drug exposure (133). In contrast, other groups have reported that aside from myeloid-based cells, non-tumour cell lines of human origin tend to be poorly responsive to taxanes in terms of TNF-α production (227,228). In addressing hypothesis (b), our finding that loss of membrane integrity does not correlate with increased TNF-α production during treatment with docetaxel (figure 3.3A and B), provides conclusive evidence that increased TNF-α from the media is originating from live cells. Data presented in figure 3.3D is supportive of this hypothesis, demonstrating an increase in TNF-α mRNA occurring prior to detectable increases in TNF-α release.

4.2 Docetaxel induces TNF-α production by a mechanism distinct from lipopolysaccharides (LPS)

An earlier study sought to characterize taxane-induced TNF-α production in murine macrophages. Reportedly, TLR4-null macrophages exhibited lower TNF-α and nitrogen oxide (NO) production in response to paclitaxel than macrophages with wildtype TLR4 levels (119). It was concluded that in murine macrophages, both paclitaxel and LPS-induced TNF-α release is TLR4-dependent. The study showed that the TLR adaptor protein MyD88 was also required, since it was observed that MyD88-knockout mice did not increase their output of TNF-α in response to either agent mentioned. It should be noted rather that MyD88-knockout mice still responded to paclitaxel and LPS with activation of mitogen-activated kinase (MAPK) and NF-κB (119). Our results suggest that TNF-α release in human tumour cells can be *independent* of the MyD88 adaptor protein, since a strong release was observed when A2780 cells that lack detectable MyD88 were treated with the structurally related taxane, docetaxel (figure 3.4B). This is in contrast to TNF-α release in response to LPS treatment of human tumour cells from this study (figure 3.11), which *exclusively* occurred in the MyD88-positive MCF-7 cells, consistent with several other studies that showed that inflammatory cytokine release in response to LPS is MyD88-dependent in ovarian cancer cell lines (207–209,229). With regards to inflammatory cytokine release in MyD88-expressing cells, we observed a discrepancy between the effects of docetaxel and LPS, since acute exposure (72 hours) to docetaxel was able to induce significant increases in TNF-α and CXCL8, while LPS stimulated comparatively

stronger increases in both of these cytokines, in addition to an increase in CXCL1 (figure 3.4), consistent with the idea that the mechanism of cytokine induction is distinct between the two agents.

Interestingly, agents that affect the cytoskeleton have also been shown to disrupt post-Golgi trafficking of cytokines (230). It is unclear whether the stabilization of microtubules by taxanes plays a role in tumour cell trafficking of TNF-α; however, it could explain why taxanes (among the drugs tested) seem to be particularly effective at promoting TNF-α release. It is believed that TNF-α in its membrane-bound (24-kDa) form (mTNF-α) is cleaved by the matrix metalloproteinase ADAM-17, to release its ectodomain, the soluble (17-kDa) form of TNF-α (sTNF-α) (192). Black et al. (192) purified and cloned ADAM-17, and suggested that this MMP specifically cleaved mTNF-α (based on results showing that inactivation of the gene in mouse cells caused a marked decrease in TNF-α production). Since then, hydroxamate-based inhibitors such as Marimastat, have been shown to inhibit the release of TNF-α as well as a variety of other shedding phenomena mediated by MMP's, suggesting that one or more MMP's are involved in TNF-α release. The findings of our study showed that Marimastat caused a partial decrease in TNF-α release induced by docetaxel in A2780 cells, but not MCF-7 cells (figure 3.6). It is possible that the lack of effect of Marimastat on TNF-α release in MCF-7 cells may be due to the involvement of other Marimastat-insensitive proteinases in the shedding of mTNF-α. Consistent with this view, it has been shown that LPS-induced cleavage of mTNF-α can be inhibited by both matrix metalloproteinase as well as serine protease inhibitors (231). This

suggests that the release of sTNF-α can be achieved by proteins other than ADAM-17, some of which are unaffected by Marimastat. It is also possible that LPS-induced TNF-α release, which was suppressed to roughly 50% by Marimastat, also involves cleavage by proteases that are not targeted by Marimastat. Alternatively, inhibition by Marimastat may have been suboptimal in this system and higher concentrations may have achieved complete inhibition of mTNF-α release by LPS. It has been reported that after stimulation of murine macrophages with LPS, mTNF-α begins to accumulate within the Golgi apparatus. Pulse chase labeling as well as subcellular fractionation studies have provided evidence of both the 24-kDa and 17-kDa forms of TNF-α within the Golgi apparatus (230). Thus, another possibility is that MMP-dependent shedding may occur at the level of the Golgi, sheltered from inhibition by Marimastat. Nevertheless, our findings provide confirmation that drug-induced TNF-α release by A2780 cells involves (at least in part) shedding of mTNF-α by MMPs. In summary, the data presented in figure 3.4A-E, figure 3.5A and B, figure 3.11A and B, together provide conclusive evidence that the mechanism of docetaxel-induced TNF-α release is distinct from the effects of lipopolysaccharides, as postulated in the first part of hypothesis (c).

4.3 Changes in the production of TNF-α and other cytokines upon selection for docetaxel resistance

Resistance to several classes of chemotherapy agents have been documented, as reviewed in chapter 1. Some studies have exemplified a phenomenon known as multidrug resistance, where tumour cells become resistant to multiple drugs. One widely documented mechanism for multidrug resistance involves the increased expression and activity of members of the ATP-Binding Cassette (ABC) family of proteins. These proteins normally serve to excrete toxins from healthy tissues such as the brain, liver, kidneys, and intestinal epithelium, and in the context of drug resistance they act as drug efflux pumps in tumour cells. One particular ABC family member P-glycoprotein (Abcb1) has been studied *in vivo* and its expression in tumour cells has been shown to influence penetration of chemotherapeutics, in some cases, through multicellular layers of solid tissue (82). It can also prevent the cellular accumulation and cytotoxicity of a variety of hydrophobic anticancer drugs, including members of the taxane, vinca alkaloid, anthracycline, and tyrosine kinase inhibitor families (141,221,222,232,233). Levels of MDR1, the gene that encodes P-gp, were found to be frequently elevated in tumours that are innately resistant to treatment, including those originating from the liver, pancreas, colon, kidney, and adrenal glands (234). Evidence that expression of P-gp contributes to drug resistance in the clinic has been reported (235,236) and its increased expression has been observed after chemotherapy treatment in breast cancer, acute lymphocytic and non-lymphocytic leukemia, neuroblastoma, pheochromocytoma, and nodular poorly differentiated

lymphoma (222,234,237,238). Though attempts to restore clinical response to anthracycline and taxane-containing regimens by the addition of a P-gp inhibitor (verapamil or tariquidar) have not shown much promise (99), the importance of cellular drug export to the clinic should not be overlooked. Firstly, it is unclear whether sufficient levels of P-gp inhibitor at the site of a patients' tumour are being achieved (99). Secondly, it's possible that P-gp-mediated drug-export in a patients' tumour actually facilitates drug penetration into the tumour core as illustrated through experimentation with a three-dimensional tumour cell model (82). Thus, counterproductively, pharmacological inhibition of P-gp in drug-resistant tumours would decrease tumour drug penetration, which could nullify any improvements in drug efficacy permitted by abrogating tumour drug-export.

Our data shows, *in vivo,* that selection of tumour cell lines for resistance to increasing concentrations of docetaxel is associated with increased expression of P-gp and a decrease in cellular accumulation of drug (figure 3.7). Treatment of docetaxel-resistant breast and ovarian tumour cells with the P-gp inhibitor Tariquidar restores cellular drug accumulation as well as drug cytotoxicity (figure 3.7B and figure 3.8). While P-gp clearly contributes to docetaxel resistance in MCF-7_{TXT10} cells, it is possible that there are other contributors to resistance at lower selection doses where P-gp expression is considerably lower (MCF-7_{TXT9}).

Aside from P-gp, whose expression continued to rise with increasing selection dose in MCF-7_{TXT} cell lines (figure 3.7A), basal levels of secreted cytokines TNF-α, CXCL1, and CXCL8 also became elevated during the selection process. Maximum output was reached at selection dose

10 (MCF-7_{TXT10}) after which levels declined toward that of wild-type cells (figure 3.9A, C, and E). A similar trend was observed in $A2780_{DXL}$ cells (figure 3.9B, D, and F), although CXCL8 levels remained constant. In an *in vivo* setting the above cytokines serve as mediators of autocrine, paracrine, and even endocrine communication between cells, acting in networks and exhibiting pleiotropic effects that depend on the nature of target cells. Depending on the tissue, a variety of cytokine-mediated processes can occur, including cell proliferation, differentiation, and apoptosis. This serves to co-ordinate homeostatic processes such as wound healing and immune responses to infection or trauma. Aberrant cytokine production has been associated with many cancers, and the chemokines CXCL8 and CXCL1, in particular, have been implicated in autocrine-based drug resistance in various tumour cell lines (157,239), as well as poor clinical outcome in ovarian cancer patients (156). More specifically, it has been shown that CXCL8 signalling, through CXCR1 and CXCR2 receptors, plays a direct role in multidrug resistance (57,154). Similarly, CXCL1 expression in response to taxane or anthracycline treatment (239) has been shown to cause drug-resistance through activation of CXCR2 (182,239,240) via autocrine signalling. Other groups also report an *in vivo* role for CXCL1 in drug resistance that involves paracrine signalling between tumour and myeloid cells (146). With respect to hypothesis (d), stating that sustained exposure to docetaxel and selection for resistance to drug causes changes in the basal production of multiple inflammatory cytokines that have been implicated in drug resistance, we can conclude that our data presented in figure 3.9A-F, provides definitive proof of this.

Previous findings from our laboratory have shown that acquisition of resistance to docetaxel in MCF-7 cells was accompanied by increased TNF-α production and secretion, as well as a decrease in cellular levels of TNFR1 (24). Blockade of TNFR2 in the resistant cells was shown to increase sensitivity to docetaxel by roughly 2-fold (24). It is unclear whether these alterations in TNF-α signalling contribute to drug resistance through modulations of P-gp expression or activity. Exogenously added TNF-α has been shown, in a variety of cell types, to stimulate P-gp expression and increased activity (241–244); however, we have observed that exposure of MCF-7 cells to TNF-α failed to promote detectable increases in P-gp at the protein level after treatment for 96 hours (see figure A1 of the appendix). At any rate, it is possible that autocrine signalling by membrane-bound TNF-α (mTNF-α) may be one of several confounding factors, since studies showing that mTNF-α, expressed by tumour cells, contributes to better survival in mice transfected with either lung or melanoma tumour cell lines (152). It is, however, unclear whether mTNF-α levels are increased in the drug-selected cell lines used in this study. Regardless, if either TNF-α or one of CXCL1 and CXCL8 promote P-gp expression in MCF-7 cells through autocrine signalling, then it would follow that this stimulus would be lost beyond selection dose 10 (15 nM docetaxel), since levels of these secreted cytokines fall considerably at higher selection doses (figure 3.9). Yet, we observe P-gp expression to increase further (figure 3.7A), which does not support a model involving cytokine-induced P-gp activity or expression. There is however, evidence to support changes in the way P-gp expression is regulated throughout the selection of MCF-7 cells for resistance to docetaxel. For example,

above selection doses 10, a regional amplification on chromosome 7 (7q21) resulting in an increased P-gp (*MDR1*) gene copy number was detected (220). This amplification may result in highly increased P-gp expression, particularly beyond selection dose 10, such that expression may no longer be driven by elevated cytokine production. Of particular relevance, recent studies have characterized TNF-α as a potent mutagen (152,245). More specifically, treatment of cultured cells with soluble TNF-α was found to cause DNA damage comparable to that of ionizing radiation (245). This, in turn, was found to cause gene amplifications, mutations, micronuclei formation, and greater chromosomal instability (245). This could be attributable to the chromosomal amplifications we observed in MCF-7_{TXT11} and MCF-7_{TXT12} cells.

In our study, we observed that despite the increased basal output of TNF-α from both breast and ovarian tumour cell lines during acquisition of docetaxel resistance, there was a diminished ability to further increase TNF-α production in response to docetaxel (figure 3.11). In contrast, LPS retained its ability to induce TNF-α production (in MCF-7_{CC10} and MCF-7_{TXT10} cells). This suggests that the mechanism for LPS-stimulated TNF-α production is distinct from that for docetaxel. Given that cellular drug accumulation in MCF-7_{TXT10} and $A2780_{DXL12}$ cells is severely diminished relative to wild-type cells, but can be restored by Tariquidar, which concomitantly causes a potentiation of the drug-induced release of TNF-α, it is our opinion that the ability of docetaxel to cause TNF-α release requires a sufficient level of drug to accumulate within the tumour cell. This is unlike TNF-α release in response to LPS, which stimulates TNF-α production by binding to extracellular TLR4 and does not require uptake into cells. Despite their

diminished ability to respond to docetaxel with increased TNF-α release, MCF-7_{TXT10} cells maintain their ability to augment TNF-α production in response to LPS, presumably through activation of TLR4 at the cell surface. Consistent with previous reports that MyD88 is an essential cellular component for LPS-induced inflammatory cytokine production, elevated TNF-α production in response to LPS treatment did not occur in $A2780_{DXL12}$ cells, which do not express detectable levels of MyD88.

4.4 Mechanistic insight into inflammatory responses induced by docetaxel

Some groups have reported that paclitaxel induces TNF-α release through the activation of TLR4, which reportedly involves a direct interaction between paclitaxel and the extracellular TLR4 adaptor protein myeloid differentiation factor 2 (MD-2) at the cell surface of macrophages (200,227). This direct activation of TLR4 appears to be the accepted mechanism by which taxanes promote TNF-α production and release in macrophages (119,133) as well as tumour cells (207,209,246), despite a lack of rigorous studies employing tumour cells. Although our findings do not discount a role for TLR4 in mediating taxane-induced TNF-α production and release from all tumour cell types, they do illustrate novel insights into the mechanism. The evidence in this study suggests that taxanes must enter MCF-7 and A2780 tumour cells to promote TNF-α production, thus not in support of a mechanism involving direct activation for TLR4 residing on the surface of the cell.

Our observation that a variety of structurally unrelated chemotherapeutic agents can induce TNF-α release suggests that drug-induced TNF-α release involves a variety of pathways that may or may not involve TLR4 activation and/or activation of some downstream pathways. Consistent with this view, a recent literature review states that "it has not been firmly established that the functional effects of paclitaxel are mediated through physical binding to TLR4" (247). One possibility is that TLR4 activation by chemotherapy drugs can involve the death-dependent release of damage-associated molecular patterns (DAMPs), a subset of alarmins, some of which can activate TLR4 and subsequent cytokine production. For example,

studies have demonstrated the release of the damage-associated molecular pattern HMGB1 after treatment with a variety of chemotherapy agents, including docetaxel (248). HMGB1 among other alarmins has been shown to activate TLR4 after HMGB1's release from necrotic cells (194). The release of DAMPs in response to docetaxel would require drug uptake into cells, consistent with our observations. Given that LPS and docetaxel were observed to induce the production of different sets of inflammatory cytokines in MCF-7 and A2780 cell lines (figure 3.4A-F), a TLR4-DAMP-dependent mechanism of inflammatory cytokine production in response to docetaxel is not expected in these cells because, if this were the case, docetaxel and LPS treatment would arguably induce the release of the same sets of cytokines. A DAMP-dependent mechanism could also involve TLRs other than TLR4, possibly involving DAMP recognition in the intracellular space. For example, TLR3, residing intracellularly, has been shown to respond to mRNA released from or associated with necrotic cells, with the production of pro-inflammatory cytokines (249). Since certain TLRs respond to DAMPs intracellularly, it is possible that drug-induced inflammatory cytokine release could involve the activation of an intracellular TLR, after displacement of an intracellular DAMP with respect to its normal distribution among specific organelles. Given the A2780 cell line does not respond to LPS, consistent with its lack of the TLR4 adaptor protein MyD88, it is expected that a DAMP-TLR-dependent drug-induced mechanism in this cell line would involve activation of a TLR other than TLR4. Drug-induced TNF-α production may also involve heat shock proteins (HSP's), as reports have shown that geldanamycin treatment, a specific inhibitor for the HSP90 family, abrogated the expression of

TNF-α in macrophages treated with paclitaxel or LPS (118). Interestingly, geldanamycin did not block microtubule stabilization by paclitaxel suggesting a mechanism independent of microtubule functionality (118). The authors of this study further suggest that paclitaxel binds to Hsp90, mediating macrophage activation (118). Further, HSP60 is thought to activate the TLR4 complex directly, eliciting a pro-inflammatory response in cells of the innate immune system (250).

Kawasaki et al. demonstrate that paclitaxel's ability to mimic the effects of LPS occurs only in murine-derived cell lines, since the expression of recombinant *murine* MD-2 in combination with either human or murine TLR4 is a requirement for inflammatory cytokine expression in response to paclitaxel (200). Consistent with this, Resman et al. failed to observe TLR4 pathway stimulation in response to docetaxel treatment of human embryonic kidney cells (HEK293). More specifically, the study reports an *inhibitory* effect of docetaxel on LPS-induced TLR4 signalling, through binding of MD-2 in HEK293 cells (228). There are many potential explanations for the lack of taxane-induced inflammatory response in human tissue. As we've seen in this study a response involving TNF-α release is clearly dose-dependent (figure 3.1). Further, the optimal dose likely differs greatly between tissues, as most studies reporting this phenomenon in macrophages or other non-malignant cell lines involve treatment with taxane concentrations roughly three orders of magnitude greater than those seen in this study with tumour cells. However, it is interesting to note that LPS-RS suppressed TNF-α production in breast tumour cells treated with LPS (figure 3.5), but in the same cell line potentiated TNF-α

release in the presence of docetaxel (figure 3.5), highlighting a distinction in the immunogenic mode of action between LPS and docetaxel. Also, interference with the *intracellular* pathway components that are activated by extracellular TLR4 ligation with LPS was shown to be insufficient for preventing docetaxel-induced TNF-α release in the MDA-MB-231 breast cancer cell line (figure 3.5B). Given the reported specificity of TAK-242 for TLR4, among the TLRs (216), and its ability to fully inhibit LPS-induced TNF-α release aswe observed here, it can be concluded that LPS is triggering TNF-α release exclusively through its interaction with TLR4. In contrast, the failure of TAK-242 to inhibit TNF-α release from breast tumour cells in response to docetaxel suggests that TLR4 is not ~~be~~ an important contributor to docetaxel-induced TNF-α release in the MB-231 cell line. Regardless, further studies are clearly warranted for a better understanding of the mechanisms at play.

4.5 Effects of LPS pretreatment on docetaxel cytotoxicity

In this study, we found that pretreatment of drug-naive and docetaxel-resistant MCF-7 cells with LPS significantly increased sensitivity to docetaxel, but this effect was absent in MyD88-deficient A2780-based cell lines (figure 3.13). Although it is unclear how LPS elicits this increased sensitization to docetaxel in MCF-7 cells, it arguably does not involve changes in P-gp activity or expression, since LPS also caused sensitization to docetaxel in drug-naive cells that lack detectable P-gp levels. It is interesting to note that the sensitizing effect of LPS in MCF-7 cells was accompanied by a cellular response involving increased cytokine production (TNF-α, CXCL8, and CXCL1). On the other hand, the inability of LPS pretreatment to cause increased sensitivity to docetaxel in MyD88-deficient A2780-based cells was associated with no effect on the production level of the aforementioned cytokines (figure 3.4 and 3.11). This suggests that the biochemical pathways that are activated by LPS, resulting in inflammatory cytokine release, are closely related to those responsible for the increased sensitization to docetaxel. Given the reported ability of TNF-α to sensitize tumour cells to taxanes (24,150), the docetaxel-sensitizing effects observed here (figure 3.13) may likewise be provoked through LPS's ability to induce TNF-α production and subsequent autocrine signalling.

It should be noted that the effects of LPS exposure on cellular sensitivity to docetaxel have been studied by other groups. Contradictory to our observations, one study shows that ligation of TLR4 with LPS caused increased *resistance* to the growth inhibitory effects of docetaxel on PC-3 prostate tumour cells (225). Likewise, other groups have shown that

activation of the TLR4 pathway in MyD88-positive SKOV3 ovarian cancer cells causes increased resistance to taxanes (207). Nevertheless, *consistent* with our results the same study showed that MyD88-negative A2780 ovarian cancer cells were unaffected upon TLR4 activation by LPS, in terms of their sensitivity to docetaxel. Thus, it would appear that part of hypothesis (c), stating that LPS may augment docetaxel cytotoxicity in the presence of docetaxel, is in some cases found to be true, but it depends on the particular tumour cell line in question. LPS exposure can either increase (as in figure 3.13A), decrease sensitivity to docetaxel (207,225), or have no effect (207) as we observed with the A2780-based cell lines (as in figure 3.13B).

LPS treatment alone was observed to have no significant effect on cell growth in either of the cell lines tested. As mentioned however, in combination with docetaxel LPS exhibited drug-sensitizing properties. It was unclear as to how LPS was causing this effect, however we report that changes in drug accumulation within the cells was not a contributing factor (figure 3.14). An interesting and potentially relevant study reported that NF-κB plays a role in protecting macrophages from LPS-induced cell death (251). They showed that when NF-κB activation was inhibited, LPS was permitted to induce cell death through activation of TLR4 and the subsequent pathway component RIP (receptor interacting protein). Interestingly, the authors concluded that LPS-induced cell death in this context did not involve the TNF-α/TNFR1 axis (251). Perhaps the activation of a similar death pathway occurs when docetaxel and LPS treatment are combined. It may be that docetaxel-induced activation of NF-κB followed by its' relocalization to the nucleus (demonstrated in Sprowl et al. (24)), compromises the ability of

NF-κB to protect against LPS-induced cell death through RIP. Although this is speculative, it *does* offer an example of TNFR1-independent cell death induced by LPS, which may explain the synergy observed between docetaxel and LPS in our MCF-7_{TXT10} cells (figure 3.13), given their relatively diminished TNFR1 levels (24). Although, there are reports of TNF-α's role in sensitizing tumour cells to taxane cytotoxicity, enhanced cell death by LPS pretreatment may not necessarily require enhanced TNF-α production. Another relevant study demonstrates LPS-induced cell death in breast epithelial and cancer cell hybrids (M13MDA435) (252). Neutralization of TNF-α signalling, induced by LPS, did not prevent cell death (252). Our observations suggest that LPS-induced sensitivity to docetaxel is associated with LPS-induced TNF-α production (figure 3.13). However, TNF-α production was not a sufficient indicator in predicting LPS-induced cell death, since LPS treatment alone, which stimulated TNF-α release, did not appear to have any cytotoxic effect on any of the cell lines. LPS-induced TNF-α production was *rather* indicative of a tumour cell lines' vulnerability to the synergistic cytotoxicity of LPS and docetaxel treatment (figure 3.13). Further study would be warranted to determine the cellular components that are necessary for the docetaxel-sensitizing effects of LPS. It is possible that MyD88 is one important determinant given that MyD88-negative A2780 cells were insensitive to these effects (figure 3.13). TLR4 activation is known to stimulate the production of transcription factors NF-κB, AP-1 and IRF3 (253). NF-κB gets activated and relocated to the nucleus only if MyD88 is recruited to the cytoplasmic domain of TLR4 (253). This means that if the cells lack the cytoplasmic MyD88 adaptor protein then they will respond

to LPS-induced TLR4 activation differently, with respect to the transcriptional regulation that occurs. NF-κB activation and relocalisation to the nucleus stimulates the production of various inflammatory cytokines, including TNF-α, which may be required after all for the drug-sensitizing effect of LPS given that TNF-α has been shown to exhibit such effects in the presence of docetaxel (24), although our data does not definitively confirm this.

4.6 *In vitro* model

As presented in this thesis, TNF-α levels were found to increase in two breast cancer cell lines (MCF-7 and MB-231) and an ovarian cancer cell line (A2780) in response to treatment with a variety of chemotherapy drugs, but most dramatically with the taxanes docetaxel and paclitaxel. Docetaxel-induced TNF-α production in MCF-7 cells has been demonstrated to have autocrine effects, potentiating the cytotoxic effect of the drug through its interaction with TNFR1 (25). Similar to docetaxel, treatment of MCF-7 cells with LPS was found to induce the production of the inflammatory cytokine TNF-α after 72 hours, but unlike docetaxel it also caused dramatic increases in CXCL1 and CXCL8 (figure 3.4 and figure 4.1).

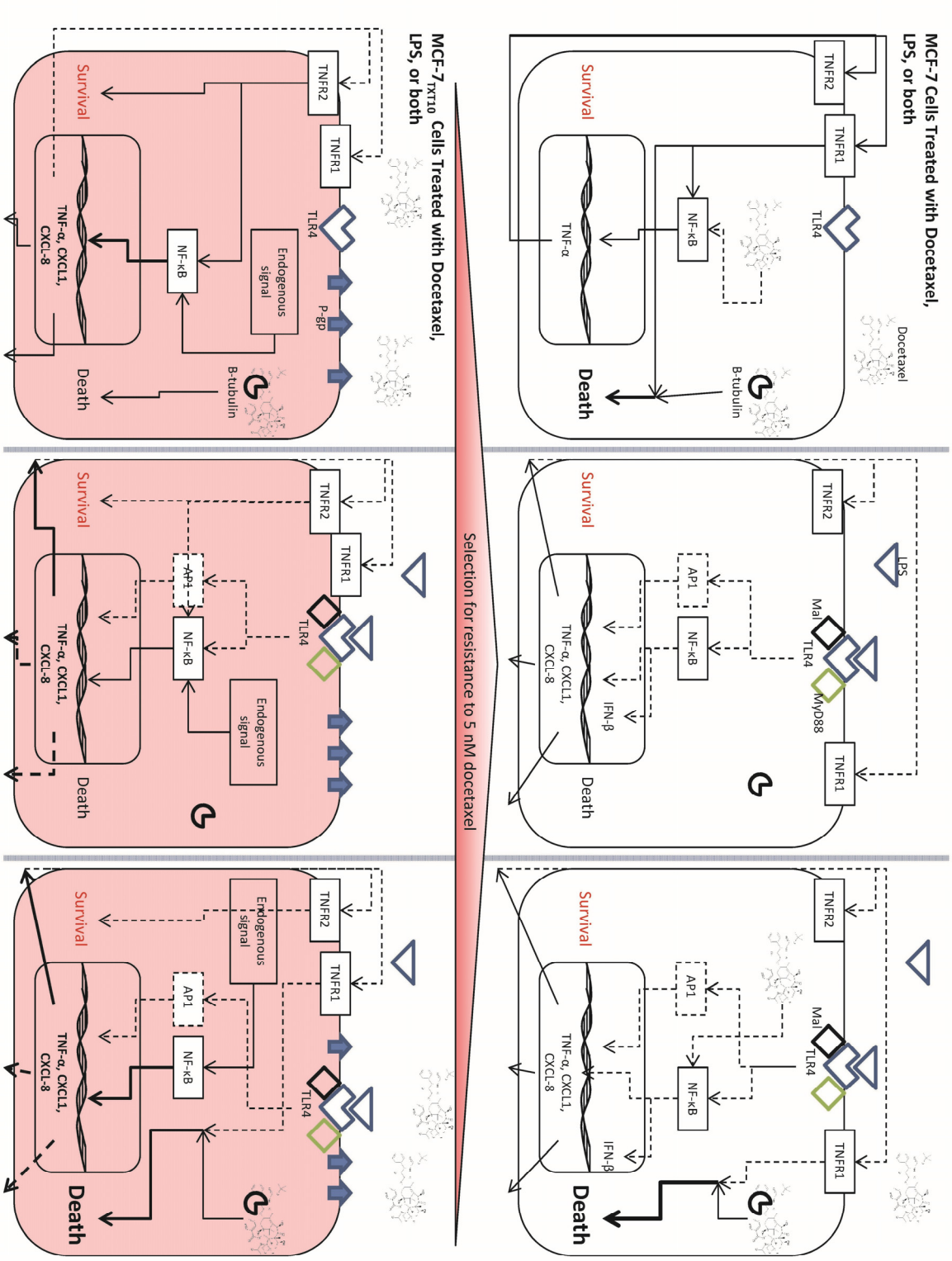
MCF-7 Cells Treated with Docetaxel, LPS, or both
MCF-7TXT10 Cells Treated with Docetaxel, LPS, or both
Selection for resistance to 5 nM docetaxel
Docetaxel
LPS
TNFR2
TNFR1
TLR4
MyD88
Mal
NF-κB
AP1
IFN-β
TNF-α
TNF-α, CXCL1, CXCL-8
B-tubulin
P-gp
Endogenous signal
Survival
Death

Figure 4.1 - Model of response to docetaxel and LPS in MCF-7 cells.

TOP - Left-most (Docetaxel): Docetaxel targets β-tubulin causing cell-cycle arrest and eventually cell death. TNF-α production is increased in response to drug (supported by data presented in figure 3.1A and C), possibly involving the activation of NF-κB, and appears to contribute to docetaxel cytotoxicity (24). *Middle* (LPS): LPS binds TLR4 and initiates the dimerization of MAL and MyD88, which causes the activation and nuclear localization of AP1, NF-κB, as proposed by others (253). This causes the increased production of TNF-alpha, CXCL1, and CXCL8 (supported by data presented in figure 3.4A, C, and E) and putative increases in IFN-β and IFN-inducible genes (253). *Right-most* (Docetaxel + LPS): The combination of docetaxel and LPS results in increased TNF-α and cell death compared to treatment with docetaxel alone (supported by data presented in figure 3.13A).

BOTTOM – Left-most (Docetaxel): Docetaxel accumulation within the cell is limited by the increased presence of the drug-export protein P-gp (supported by data presented in figure 3.7A and B), TNFR1 levels have diminished relative to co-culture control (drug-naive) cells (24), and there is a constitutive increase in the expression of TNF-α, CXCL1, and CXCL8 (supported by data presented in figures 3.9A, 3.9C, 3.9E, and A4), as a result of the selection for survival in the presence of up to 5 nM of docetaxel *(left, middle and right)*. *Middle* (LPS): LPS treatment causes the increased production of TNF-α (supported by data presented in figure 3.11A), and putative increases in the production of CXCL1, CXCL8 (as observed in drug-naive MCF-7 (figure 3.4C and E), IFN-β, and IFN-inducible genes, presumably through the activation and nuclear localization of AP1 and NF-kB transcription factors (253). *Right-most* (Docetaxel + LPS): The combination of LPS and docetaxel causes increased release of TNF-α and cell death compared to treatment with docetaxel alone (supported by data presented in figure 3.13A).

Along with increased P-gp expression, selection for survival in the presence of increasing concentrations of docetaxel led to a greater capacity for docetaxel extrusion and thus lower accumulation of the drug after treatments in MCF-7_{TXT10} and $A2780_{DXL12}$ cells (figure 3.7). To assess whether intracellular accumulation of drug is an important factor in docetaxel-induced TNF-α release, TNF-α release was assessed in response to docetaxel treatment, with and without the addition of a specific inhibitor for P-gp (tariquidar). Results showed that inhibition of P-gp-mediated drug export, allowed a potentiation of docetaxel-induced TNF-α production in the drug-resistant cells (figure 3.12). This is consistent with docetaxel-induced TNF-α production requiring drug to accumulate in tumour cells.

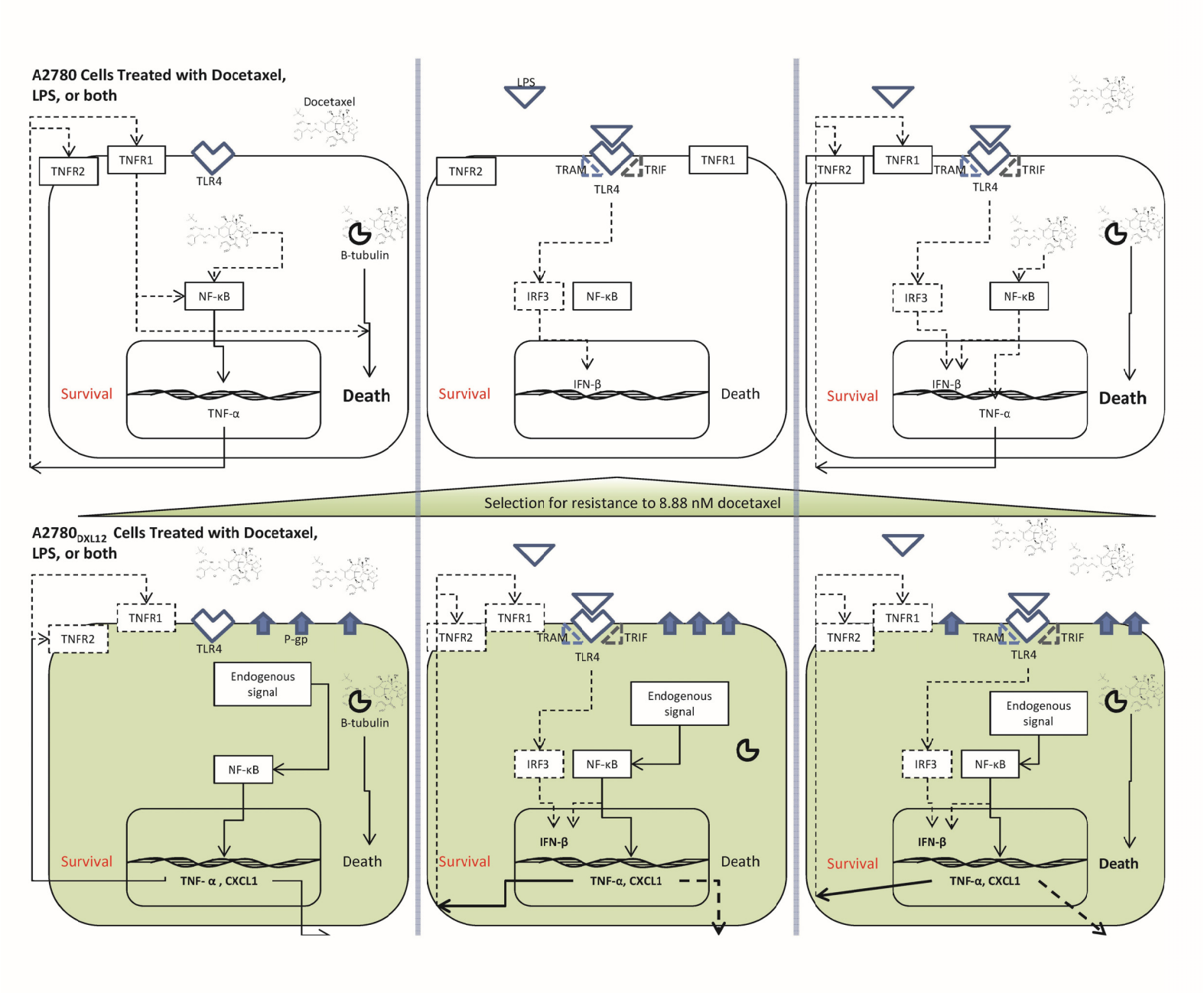
A2780 Cells Treated with Docetaxel, LPS, or both
Docetaxel
LPS
TNFR2
TNFR1
TLR4
TRAM
TRIF
B-tubulin
NF-κB
IRF3
Survival
Death
TNF-α
IFN-β
Selection for resistance to 8.88 nM docetaxel
A2780DXL12 Cells Treated with Docetaxel, LPS, or both
P-gp
Endogenous signal
TNF- α , CXCL1
TNF-α, CXCL1

Figure 4.2 - Model of response to docetaxel and LPS for A2780 cells.

TOP - *Left-most* (Docetaxel): Docetaxel targets β-tubulin causing cell-cycle arrest and eventually cell death. TNF-α release is increased in response to drug (supported by data presented in figure 3.1B and D)) and may or may not contribute to docetaxel cytotoxicity as with MCF-7 cells. *Middle* (LPS): In speculation, LPS binds TLR4 and initiates the dimerization of TRIF and TRAM, rather than MyD88 and Mal, which causes the activation and nuclear localization of IRF3 transcription factor (253). This causes the increased production of IFN-β and IFN-inducible genes (253). *Right-most* (Docetaxel + LPS): Treatment with LPS, in the presence of docetaxel, does not result in any increased sensitivity to docetaxel (supported by data presented in figure 3.13B).

BOTTOM – Left-most (Docetaxel): Docetaxel accumulation within the cell is limited by the increased presence of the drug-export protein P-gp (supported by data presented in figure 3.8B), TNFR1 levels may have diminished relative to co-culture control cells (as with MCF-7 cells), and there is a constitutive increase in the expression of TNF-α and CXCL1 (supported by data presented in figures 3.9B, 3.9F) as a result of the selection for survival in the presence of up to 8.88 nM of docetaxel (*left, middle and right*). *Middle* (LPS): In speculation, LPS treatment causes the increased production of IFN-β and IFN-inducible genes presumably through the activation and nuclear localization of IRF3 (253). *Right-most* (Docetaxel + LPS): Treatment with LPS, in the presence of docetaxel, does not cause increased sensitivity to docetaxel (supported by data presented in figure 3.13B).

β-Tubulin

Docetaxel

LPS (Lipopolysaccharides)

TLR4 (Toll-like Receptor 4)

MyD88 (Myeloid Differentiation Factor 88)

Mal (MyD88-adapter-like) a.k.a TIRAP (TIR domain containing adaptor protein)

TRAM (Toll-like Receptor Adaptor Molecule 2)

TRIF (TIR-domain-containing adapter-inducing interferon-β)

P-gp (P-glycoprotein)

1. Relative weight of arrows indicates the relative magnitude of the signal.
2. Arrows indicate interaction/activation of a protein or gene promoter.
3. Hashed boarders or arrows indicate putative expression of a protein, or interaction/activation/expression of a protein or gene promoter, respectively.

Selection for survival in the presence of docetaxel also led to changes in the constitutive production of TNF-α, CXCL8, and CXCL1 in MCF-7 breast cancer cells (figure 3.9), and likewise TNF-α and CXCL1 in A2780 ovarian cancer cells. It is interesting to note that cytokine profiles for short-term exposure (figure 3.4), were different than those observed after long-term selection in increasing concentrations of docetaxel (figure 3.9). Moreover, it was confirmed that long-term selection resulted in increased cytokine production beyond 5 days after drug was removed from media, indicating a stable change to transcriptional expression patterns.

Since we observed increased TNF-α levels after acute treatment of drug-naive cancer cells with docetaxel, and increased TNF-α and CXCL1 protein expression (along with increases in TNF-α (24) and CXCL1 (figure A4) mRNA in MCF-7) in both MCF-7 and A2780 cell lines upon prolonged drug selection, it is possible that increased CXCL1 production is an autocrine response to elevations in TNF-α concentrations. Supporting this view, it has been shown that, through paracrine signalling, breast cancer cells responded to TNF-α produced by endothelial cells, with increased CXCL1 and CXCL2 production within a tumour cell-implanted mouse model (146). Induction of CXCL1 expression and release after exposure to recombinant TNF-α has also been demonstrated *in vitro*, albeit from human vascular endothelial cells (254). Specifically, it was suggested that TNF-α promotes JNK-mediated CXCL1 mRNA expression, and secretory processes mediated by p38 MAPK and PI-3K (254). The question remains as to how important cytokine signalling pathways are to cancer incidence, tumour progression, and treatment outcome in cancer patients. As reviewed by Acharyya et al., amplification of 4q21 occurs in

breast cancer and promotes the upregulated expression of fifteen genes including cytokines CXCL1-8 (255). Elevated expression of CXCL1, however, can occur without gene amplification (256). Fluorescence in situ hybridization (FISH) studies have shown that CXCL1/2 genes were amplified in 7.5% of primary breast tumours and 19.9% of metastases (146). The fact that drug-induced TNF-α production can occur in cultured tumour cells after 48 hours, (24,142) and as we've shown in chapter 3, that long-term drug exposure and selection for drug resistance can give rise to the increased production of a variety of inflammatory cytokines (TNF-α, CXCL1, and CXCL8), presents a number of potential clinical implications. Given that *in vivo* mouse studies have shown that tumour-derived cytokine release can have significant effects on tumour behaviour (146,151,152,156,185), it is likely that tumour cell exposure to chemotherapy agents, such as docetaxel, stimulates the production of inflammatory cytokines (including TNF-α, CXCL1, and CXCL8), which in turn affect tumour progression via their ability to induce immune cell migration (146,151,185), angiogenesis (151,156), and/or enhanced survival pathways (24,57,157,165) within a patients' tumour microenvironment while having significant consequences for treatment outcome. The net physiological effect of TNF-α and other cytokines that become increasingly produced in response to docetaxel treatment is thus an important area of study. The answer to whether this is good or bad for a patient receiving treatment likely depends on many factors, including which tissue these inflammatory signals are able to target. It is unclear whether TNF-α produced by the tumour has any significant impact on tissue outside of the tumour microenvironment. Insight into the possibility that

tumour-derived cytokines can yield systemic physiological effects and what the nature of these effects would be is of great importance in determining how to better manage patients and eradicate tumours that emit inflammatory signals.

4.7 Conclusions

We conclude that both drug dose as well as exposure time are important factors affecting the release of TNF-α and other inflammatory cytokines (CXCL1 and CXCL8) from breast and ovarian tumour cells. We showed that a variety of other chemotherapy agents share the ability to stimulate the release of TNF-α in tumour cell lines originating from distinct tissues. This suggests that the biochemical pathways activated in the release of TNF-α are associated with cell death, whether through similar or very distinct mechanisms of action. Our data suggests that docetaxel-induced TNF-α release from MCF-7 cells is an active cellular process, since it involves an increase in TNF-α mRNA transcription, and does not positively correlate with loss of membrane integrity. We showed that docetaxel's ability to induce the secretion of cytokines in tumour cells, originating from the breast and ovaries, is distinct from LPS in a variety of ways. Firstly, docetaxel does not stimulate the release of the same inflammatory cytokines as LPS, from MCF-7 breast tumour cells. Secondly, docetaxel stimulated TNF-α release from MyD88-negative ovarian tumour cells, where LPS could not. Thirdly, docetaxel appears to require cellular uptake by tumour cells in order to stimulate increased TNF-α release, whereas LPS appears to stimulate the release via an extracellular receptor. We also conclude that sustained drug exposure and the acquisition of resistance to docetaxel causes changes in the basal production levels of TNF-α, CXCL1, and CXCL8, all of which have been shown to affect tumour growth through either autocrine (involving only tumour) or paracrine signaling (involving both the tumour and other cell types in the tumour microenvironment). As many

research groups continue to uncover the importance of extracellular factors originating from tumour cells in the treatment of cancer, we demonstrate that tumour inflammatory pathways can be exploited to augment the cytotoxic effect of docetaxel in tumour cells. Specifically, we are the first to show that stimulation of the TLR4 pathway in breast tumour cells can improve the cytotoxic effect of docetaxel.

4.8 Future directions

There is much to be explored, particularly related to the cellular receptors involved in triggering cytokine production from tumour cells in response to chemotherapeutics. Also, of value is a better understanding of the structural characteristics that give drugs their immunogenic potential, causing either the induction of inflammatory cytokines or the release of alarmins. Future experimentation is necessary to further characterize the full breadth of signals released by tumour cells during exposure to chemotherapy drugs, as well as the roles that these signals have in stimulating both autocrine, as well as paracrine signalling pathways involving both tumour and non-tumour cells within the tumour microenvironment.

The diametrically opposing effects of TNF-α observed by Ardestani et al. (152) in a xenograft mouse model, whereby TNF-α in its membrane-bound form expressed by the tumour contributes to tumour supression, yet TNF-α in its soluble form released by the tumour contributes to the chemotactic accumulation of myeloid cells at the site of the tumour and its progression, are remarkably relevant and worthy of further consideration. Improving methodologies for measuring TNF-α as both a soluble and membrane-bound isoform in our *in vitro* models, and discerning between the level of each, would be a worthy goal. This would allow the determination of whether membrane-bound TNF-α levels change within a tumour cell during exposure to chemotherapy agents and also allow further characterization of the effects of each TNF-α isoform on tumour cell survival in the context of chemotherapy treatment and drug resistance.

Given the effect of docetaxel on the release of inflammatory signals from tumour cells, it may also be of value to determine whether this in turn can affect the behaviour of other cell types that might be found within the tumour microenvironment. A simplified, *in vitro*, system could be used where culture media from drug-treated (acute or sustained drug treatment) tumour cells is administered to other cell types that might be found in the tumour microenvironment, such as myeloid cells, in order to assess behavioural changes such as differentiation to an endothelial phenotype. A related study assessed this by measuring the presence of the endothelial cell marker VEGFR2/Flk-1, which was found to increase after tumour-associated myeloid cells were exposed to low levels of TNF-α, as reported by Li et al. (151). Thus, it would be valuable to assess whether *drug-induced* changes in cytokine release could stimulate differentiation of this nature in myeloid cells through paracrine action.

In light of our data (figure 3.9) showing increased basal production of TNF-α and CXCL1 in A2780 cells and increases of TNF-α, CXCL1 and CXCL8 in MCF-7 cells upon acquisition of resistance to docetaxel, an assessment of the potential roles of autocrine signaling in the drug-resistant phenotype would be of value. More specifically, a drug sensitivity assay in the presence or absence of conditions that block cytokine signaling through CXCR1 and CXCR2 (cognate receptors of CXCL1 and CXCL8) could be used as an assessment tool. Likewise, TNFR2 blockade with a receptor neutralizing antibody (as performed in Sprowl et al. 2012) could be used in A2780 cells to determine whether there is a similar contribution of TNF-α autocrine signaling in the drug-resistant phenotype exhibited by $A2780_{DXL12}$, as seen with previous studies

in MCF-7_{TXT10} (24). If CXCL1 or CXCL8 are found to be contributors in the drug-resistant phenotype, then the potential effect that these cytokines have on P-gp expression or activity should be assessed, given our data (figure 3.8) suggesting that P-gp-mediated drug export is a primary mode of drug resistance in both MCF-7_{TXT10} and $A2780_{DXL12}$.

As mentioned, TLR4 is an ideal target for the activation of inflammatory pathways and its activation has become a promising strategy for therapeutic intervention and the development of vaccine adjuvants (257). Although LPS is the most studied ligand for TLR4, its known toxicity in humans limits its clinical use as a vaccine adjuvant (257). However, much of the LPS structure that is responsible for its toxicity has proven to be unnecessary for TLR4 activation, and research has shown that the lipid A component is sufficient (258). This knowledge has prompted the study of less toxic lipid A-based TLR4 agonists such as monophosphoryl lipid A (MPLA) (259), the synthetic MPLA derivative glycopyranosyl lipid A (GLA) and synthetic MPLA mimetics, such as aminoalkyl glucosaminide 4-phosphates (260). All three of these TLR4 agonists are currently used in a variety of adjuvant formulations (257). MPLA, in particular, is part of the AS04 adjuvant formulation, which is ”one of the most successful vaccine adjuvants to date" (257). Along with other TLR4 agonists, MPLA is the subject of several clinical studies related to vaccine response and it is found in vaccines currently used for the prevention of a variety of infections (259,261–263), and cancer (257,264,265). These agonists may be worthy of assessment for their ability to augment chemotherapy response in cancer patients. *In vitro* study would be required to first determine whether these more

tolerable TLR4 agonists, currently being used clinically (257), can potentiate the cytotoxic effects of docetaxel and/or other chemotherapy agents in tumour cells, as we've shown to be the case with LPS. In the event that any of these more clinically tolerable TLR4 agonists are observed to augment chemotherapy cytotoxicity, further research focussed on identifying the cellular components that confer a tumour's sensitivity to the TLR4 agonist-based augmented chemotherapy response would be of value. In addition, the development of methods that can easily and reliably detect the expression of TLR4 and other important pathway components (ie. potentially MyD88) in patient tumour biopsies would be necessary to identify patients that would benefit from treatment with such an agonist. This is important because it is likely that not all patients would benefit from the addition of a TLR4 agonist, given our data that A2780 ovarian cancer cells showed no detectable difference in sensitivity to the addition of a TLR4 agonist when treated with docetaxel (figure 3.13B), which was supported by other reports that showed MyD88-negative ovarian cancer cell lines to be unaffected by TLR4 pathway stimulation (207) in the presence of a taxane. It would also be of value to determine which chemotherapy agents may synergize with TLR4 agonists and which do not. Along with the potential augmentation in chemotherapy efficacy resulting from stimulation of TLR4 on tumour cells, it is possible that a TLR4 agonist may also yield improvements in chemotherapy efficacy through its effects on healthy immune cells. In support of this, is evidence that TLR4 activity in tumour-associated dendritic cells is fundamental to host tumour immunity and optimal chemotherapy efficacy (194). In summary, further studies are warranted to identify clinically tolerable agents

and the cellular factors that permit TLR4-mediated sensitization to docetaxel. This may help identify new approaches to treat certain drug-resistant tumours in patients. It follows that improving our understanding of the role of cytokine signalling during chemotherapy will allow researchers to design new therapy regimens that can alter particular signalling networks in order to favour a better treatment outcome. Improving efficacy of treatment may require the discovery of reliable targets for either circumventing the deleterious effects of cytokine production within the host and/or exploiting the ability of cytokine signalling pathways to promote tumour cell death, with or without the activation of immune responses in cancer patients, depending on the nature of their disease and the health of their immune system.

References

1. 10 Must-Know 2015 Global Cancer Facts [Internet]. American Cancer Society. 2015 [cited 2016 Sep 22]. Available from: http://www.cancer.org/research/acsresearchupdates/more/10-must-know-2015-global-cancer-facts
2. Coleman MP, Quaresma M, Berrino F, Lutz JM, De Angelis R, Capocaccia R, et al. Cancer survival in five continents: a worldwide population-based study (CONCORD). Lancet Oncol. 2008;9(8):730–56.
3. Anderson BO, Yip CH, Smith RA, Shyyan R, Sener SF, Eniu A, et al. Guideline implementation for breast healthcare in low-income and middle-income countries: Overview of the breast health global initiative Global Summit 2007. In: Cancer. 2008. p. 2221–43.
4. Carlson RW, Allred DC, Anderson BO, Burstein HJ, Carter WB, Edge SB, et al. Breast cancer. Clinical practice guidelines in oncology. J Natl Compr Canc Netw [Internet]. 2009;7(2):122–92. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19768876%5Cnhttp://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19200416
5. Martin M, Pienkowski T, Mackey J, Pawlicki M, Guastalla J-P, Weaver C, et al. Adjuvant docetaxel for node-positive breast cancer. N Engl J Med. 2005;352(22):2302–13.
6. Martín M, Seguí M a, Antón A, Ruiz A, Ramos M, Adrover E, et al. Adjuvant docetaxel for high-risk, node-negative breast cancer. N Engl J Med. 2010;363(23):2200–10.
7. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Davidson NE, et al. Trastuzumab plus Adjuvant Chemotherapy for Operable HER2-Positive Breast Cancer. N Engl J Med [Internet]. 2005;353(16):1673–84. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa052122
8. Citron ML, Berry DA, Cirrincione C, Hudis C, Winer EP, Gradishar WJ, et al. Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of Intergroup Trial C9741/Cancer and Leukemia. J Clin Oncol [Internet]. 2003 May 15 [cited 2015 Dec 31];21(8):1431–9. Available from: http://jco.ascopubs.org/content/21/8/1431.abstract
9. Coombes RC, Bliss JM, Wils J, Morvan F, Espié M, Amadori D, et al. Adjuvant Cyclophosphamide, Methotrexate, and Fluorouracil Versus Fluorouracil, Epirubicin, and

Cyclophosphamide Chemotherapy in Premenopausal Women with Axillary Node-Positive Operable Breast Cancer: Results of a Randomized Trial. J Clin Oncol. 1996;14(1):35–45.

10. Aisner J, Weinberg V, Perloff M, Weiss R, Perry M, Korzun A, et al. Chemotherapy versus chemoimmunotherapy (CAF v CAFVP v CMF each ?? MER) for metastatic carcinoma of the breast: A CALGB study. J Clin Oncol. 1987;5(10):1523–33.

11. Martin M, Villar A, Sole-Calvo A, Gonzalez R, Massuti B, Lizon J, et al. Doxorubicin in combination with fluorouracil and cyclophosphamide (i.v. FAC regimen, day 1, 21) versus methotrexate in combination with fluorouracil and cyclophosphamide (i.v. CMF regimen, day 1, 21) as adjuvant chemotherapy for operable breast cancer: a study by the GEICAM group. Ann Oncol [Internet]. Oxford University Press; 2003 Jun 1 [cited 2017 Aug 18];14(6):833–42. Available from: https://academic.oup.com/annonc/article-lookup/doi/10.1093/annonc/mdg260

12. Jones SE, Savin MA, Holmes FA, O'Shaughnessy JA, Blum JL, Vukelja S, et al. Phase III trial comparing doxorubicin plus cyclophosphamide with docetaxel plus cyclophosphamide as adjuvant therapy for operable breast cancer. J Clin Oncol. 2006;24(34):5381–7.

13. Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Rolski J, et al. Phase III Randomized Trial Comparing Doxorubicin and Cyclophosphamide Followed by Docetaxel (AC→T) with Doxorubicin and Cyclophosphamide Followed by Docetaxel and Trastuzumab (AC→TH) with Docetaxel, Carboplatin and Trastuzumab (TCH) in Her2neu Positive E…. Cancer Res [Internet]. 2014 [cited 2017 Aug 7];69(24 Supplement). Available from: http://cancerres.aacrjournals.org/content/69/24_Supplement/62

14. Edwardson D, Chewchuk S, Parissenti AM. Resistance to Anthracyclines and Taxanes in Breast Cancer. In: Breast Cancer Metastasis and Drug Resistance [Internet]. New York, NY: Springer New York; 2013 [cited 2017 May 22]. p. 227–47. Available from: http://link.springer.com/10.1007/978-1-4614-5647-6_13

15. Paridaens RJ, Dirix LY, Beex L V, Nooij M, Cameron DA, Cufer T, et al. Phase III study comparing exemestane with tamoxifen as first-line hormonal treatment of metastatic breast cancer in postmenopausal women: the European Organisation for Research and Treatment of Cancer Breast Cancer Cooperative Group. J Clin Oncol [Internet]. American Society of Clinical Oncology; 2008 Oct 20 [cited 2017 Aug 18];26(30):4883–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18794551

16. Jordan VC, Brodie AMH. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids [Internet]. 2007 Jan [cited 2017 Aug 18];72(1):7–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17169390

17. Pommerenke E, Mattern J, Volm M. Modulation of doxorubicin-toxicity by tamoxifen in multidrug-resistant tumor cells in vitro and in vivo. J Cancer Res Clin Oncol [Internet].

1994 [cited 2017 Aug 18];120(7):422–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8188736

18. Hembruff SL, Laberge ML, Villeneuve DJ, Guo B, Veitch Z, Cecchetto M, et al. Role of drug transporters and drug accumulation in the temporal acquisition of drug resistance. BMC Cancer [Internet]. 2008;8:318. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18980695

19. Veitch ZW, Guo B, Hembruff SL, Bewick AJ, Heibein AD, Eng J, et al. Induction of 1C aldoketoreductases and other drug dose-dependent genes upon acquisition of anthracycline resistance. Pharmacogenet Genomics [Internet]. 2009 Jun [cited 2017 May 21];19(6):477–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19440163

20. Weiss RB. The anthracyclines: will we ever find a better doxorubicin? Semin Oncol. 1992;19(6):670–86.

21. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev. 2004;56(2):185–229.

22. Roninson IB, Broude E V., Chang B-D. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat [Internet]. 2001 Oct [cited 2017 Aug 20];4(5):303–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11991684

23. Orr GA, Verdier-Pinard P, McDaid H, Horwitz SB. Mechanisms of Taxol resistance related to microtubules. Oncogene [Internet]. 2003 Oct 20 [cited 2017 May 21];22(47):7280–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14576838

24. Sprowl JA, Reed K, Armstrong SR, Lanner C, Guo B, Kalatskaya I, et al. Alterations in tumor necrosis factor signaling pathways are associated with cytotoxicity and resistance to taxanes: a study in isogenic resistant tumor cells. Breast Cancer Res [Internet]. 2012 Jan [cited 2016 Jan 8];14(1):R2. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3496117&tool=pmcentrez&rendertype=abstract

25. Ueda K, Cornwell MM, Gottesman MM, Pastan I, Roninson IB, Ling V, et al. The mdr1 gene, responsible for multidrug-resistance, codes for P-glycoprotein. Biochem Biophys Res Commun [Internet]. 1986 Dec 30 [cited 2017 May 21];141(3):956–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2880583

26. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer [Internet]. 2005 Apr [cited 2017 Aug 20];5(4):275–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15803154

27. Ise W, Heuser M, Sanders K, Beck J, Gekeler V. P-glycoprotein-associated resistance to

taxol and taxotere and its reversal by dexniguldipine-HCl, dexverapamil-HCl, or cyclosporin A. Int J Oncol [Internet]. 1996 May 1 [cited 2017 Aug 20]; Available from: http://www.spandidos-publications.com/10.3892/ijo.8.5.951

28. Guo B, Villeneuve DJ, Hembruff SL, Kirwan AF, Blais DE, Bonin M, et al. Cross-resistance studies of isogenic drug-resistant breast tumor cell lines support recent clinical evidence suggesting that sensitivity to paclitaxel may be strongly compromised by prior doxorubicin exposure. Breast Cancer Res Treat [Internet]. 2004 May [cited 2016 Jan 14];85(1):31–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15039596

29. Gianni L, Viganò L, Locatelli A, Capri G, Giani A, Tarenzi E, et al. Human pharmacokinetic characterization and in vitro study of the interaction between doxorubicin and paclitaxel in patients with breast cancer. J Clin Oncol [Internet]. 1997 May [cited 2017 May 21];15(5):1906–15. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9164201

30. Paridaens R, Biganzoli L, Bruning P, Klijn JGM, Gamucci T, Houston S, et al. Paclitaxel Versus Doxorubicin as First-Line Single-Agent Chemotherapy for Metastatic Breast Cancer: A European Organization for Research and Treatment of Cancer Randomized Study With Cross-Over. J Clin Oncol [Internet]. 2000 Feb 14 [cited 2017 Aug 20];18(4):724–724. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10673513

31. Desai A, Mitchison TJ. MICROTUBULE POLYMERIZATION DYNAMICS. Annu Rev Cell Dev Biol [Internet]. 1997 Nov [cited 2017 Aug 20];13(1):83–117. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9442869

32. Sullivan KF, Cleveland DW. Identification of conserved isotype-defining variable region sequences for four vertebrate beta tubulin polypeptide classes. Proc Natl Acad Sci U S A [Internet]. 1986 Jun [cited 2017 Aug 20];83(12):4327–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3459176

33. MacRae TH. Tubulin post-translational modifications--enzymes and their mechanisms of action. Eur J Biochem [Internet]. 1997 Mar 1 [cited 2017 Aug 20];244(2):265–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9118990

34. Curmi PA, Noguès C, Lachkar S, Carelle N, Gonthier MP, Sobel A, et al. Overexpression of stathmin in breast carcinomas points out to highly proliferative tumours. Br J Cancer [Internet]. 2000 Jan 10 [cited 2017 Aug 20];82(1):142–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10638981

35. Derry WB, Wilson L, Khan IA, Ludueña RF, Jordan MA. Taxol Differentially Modulates the Dynamics of Microtubules Assembled from Unfractionated and Purified β-Tubulin Isotypes †. Biochemistry [Internet]. 1997 Mar 25 [cited 2017 Aug 20];36(12):3554–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9132006

36. Pascal Verdier-Pinard ‡, Fang Wang §, Laura Martello ‡,‖, Berta Burd ‡, George A. Orr ‡

and, Susan Band Horwitz* ‡. Analysis of Tubulin Isotypes and Mutations from Taxol-Resistant Cells by Combined Isoelectrofocusing and Mass Spectrometry†. American Chemical Society ; 2003 [cited 2017 Aug 20]; Available from: http://pubs.acs.org/doi/abs/10.1021/bi027293o

37. Burkhart CA, Kavallaris M, Band Horwitz S. The role of beta-tubulin isotypes in resistance to antimitotic drugs. Biochim Biophys Acta [Internet]. 2001 [cited 2017 Aug 20];1471(2):O1-9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11342188

38. Shalli K, Brown I, Heys SD, Schofield AC. Alterations of beta-tubulin isotypes in breast cancer cells resistant to docetaxel. FASEB J [Internet]. Federation of American Societies for Experimental Biology; 2005 Aug [cited 2017 May 21];19(10):1299–301. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15946994

39. Berrieman HK, Lind MJ, Cawkwell L. Do β-tubulin mutations have a role in resistance to chemotherapy? Lancet Oncol [Internet]. 2004 Mar [cited 2017 May 21];5(3):158–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15003198

40. Gonzalez-Garay ML, Chang L, Blade K, Menick DR, Cabral F. A beta-tubulin leucine cluster involved in microtubule assembly and paclitaxel resistance. J Biol Chem [Internet]. 1999 Aug 20 [cited 2017 Aug 20];274(34):23875–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10446152

41. Cabral FR, Brady RC, Schibler MJ. A mechanism of cellular resistance to drugs that interfere with microtubule assembly. Ann N Y Acad Sci [Internet]. 1986 [cited 2017 Aug 20];466:745–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2873781

42. Maeno K, Ito K, Hama Y, Shingu K, Kimura M, Sano M, et al. Mutation of the class I beta-tubulin gene does not predict response to paclitaxel for breast cancer. Cancer Lett [Internet]. 2003 Jul 30 [cited 2017 Aug 20];198(1):89–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12893435

43. Fan W. Possible mechanisms of paclitaxel-induced apoptosis. Biochem Pharmacol [Internet]. 1999 Jun 1 [cited 2017 Aug 20];57(11):1215–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10230764

44. Srivastava RK, Srivastava AR, Korsmeyer SJ, Nesterova M, Cho-Chung YS, Longo DL. Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. Mol Cell Biol [Internet]. 1998 Jun [cited 2017 Aug 21];18(6):3509–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9584191

45. Haldar S, Basu A, Croce CM. Bcl2 is the guardian of microtubule integrity. Cancer Res [Internet]. 1997 Jan 15 [cited 2017 Aug 20];57(2):229–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9000560

46. Rodi DJ, Janes RW, Sanganee HJ, Holton RA, Wallace B., Makowski L. Screening of a library of phage-displayed peptides identifies human Bcl-2 as a taxol-binding protein 1 1Edited by I. A. Wilson. J Mol Biol [Internet]. 1999 Jan 8 [cited 2017 Sep 2];285(1):197–203. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9878399

47. Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. Cancer Res [Internet]. 1999 Apr 1 [cited 2017 Sep 2];59(7 Suppl):1693s–1700s. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10197582

48. Greenberger LM, Sampath D. Resistance To Taxanes. In: Teicher B, editor. Cancer Drug Discovery and Development: Cancer Drug Resistance. Totowa: Humana Press Inc; 2012. p. 329–58.

49. Huang Y, Ray S, Reed JC, Ibrado AM, Tang C, Nawabi A, et al. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. Breast Cancer Res Treat [Internet]. 1997 Jan [cited 2017 Sep 2];42(1):73–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9116321

50. Sui M, Huang Y, Park BH, Davidson NE, Fan W. Estrogen Receptor Mediates Breast Cancer Cell Resistance to Paclitaxel through Inhibition of Apoptotic Cell Death. Cancer Res [Internet]. 2007 Jun 1 [cited 2017 Sep 2];67(11):5337–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17545614

51. Henderson IC, Berry DA, Demetri GD, Cirrincione CT, Goldstein LJ, Martino S, et al. Improved Outcomes From Adding Sequential Paclitaxel but Not From Escalating Doxorubicin Dose in an Adjuvant Chemotherapy Regimen for Patients With Node-Positive Primary Breast Cancer. J Clin Oncol [Internet]. 2003 Mar 15 [cited 2017 Sep 2];21(6):976–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12637460

52. Lippman ME, Allegra JC, Thompson EB, Simon R, Barlock A, Green L, et al. The Relation between Estrogen Receptors and Response Rate to Cytotoxic Chemotherapy in Metastatic Breast Cancer. N Engl J Med [Internet]. Massachusetts Medical Society ; 1978 Jun 13 [cited 2017 Sep 2];298(22):1223–8. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM197806012982203

53. Maehara Y, Emi Y, Sakaguchi Y, Kusumoto T, Kakeji Y, Kohnoe S, et al. Estrogen-receptor-negative breast cancer tissue is chemosensitive in vitro compared with estrogen-receptor-positive tissue. Eur Surg Res [Internet]. 1990 [cited 2017 Sep 2];22(1):50–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2379525

54. Wang Z, Goulet R, Stanton KJ, Sadaria M, Nakshatri H. Differential effect of anti-apoptotic genes Bcl-xL and c-FLIP on sensitivity of MCF-7 breast cancer cells to paclitaxel and docetaxel. Anticancer Res [Internet]. [cited 2017 Sep 2];25(3c):2367–79. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16080463

55. Garrison JB, Samuel T, Reed JC. TRAF2-binding BIR1 domain of c-IAP2/MALT1 fusion protein is essential for activation of NF-κB. Oncogene [Internet]. 2009 Apr 2 [cited 2017 Sep 2];28(13):1584–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19234489

56. Hettmann T, DiDonato J, Karin M, Leiden JM. An essential role for nuclear factor kappaB in promoting double positive thymocyte apoptosis. J Exp Med [Internet]. 1999 Jan 4 [cited 2017 Sep 2];189(1):145–58. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9874571

57. Shi Z, Yang W-M, Chen L-P, Yang D-H, Zhou Q, Zhu J, et al. Enhanced chemosensitization in multidrug-resistant human breast cancer cells by inhibition of IL-6 and IL-8 production. Breast Cancer Res Treat [Internet]. 2012 Aug 26 [cited 2016 Jan 8];135(3):737–47. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22923236

58. Montagut C, Tusquets I, Ferrer B, Corominas JM, Bellosillo B, Campas C, et al. Activation of nuclear factor- B is linked to resistance to neoadjuvant chemotherapy in breast cancer patients. Endocr Relat Cancer [Internet]. 2006 Jun 1 [cited 2017 Sep 2];13(2):607–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16728586

59. Hasegawa S, Miyoshi Y, Egawa C, Ishitobi M, Taguchi T, Tamaki Y, et al. Prediction of response to docetaxel by quantitative analysis of class I and III beta-tubulin isotype mRNA expression in human breast cancers. Clin Cancer Res [Internet]. 2003 Aug 1 [cited 2017 Sep 2];9(8):2992–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12912947

60. Paradiso A, Mangia A, Chiriatti A, Tommasi S, Zito A, Latorre A, et al. Biomarkers predictive for clinical efficacy of taxol-based chemotherapy in advanced breast cancer. Ann Oncol [Internet]. 2005 May 1 [cited 2017 Sep 2];16(suppl_4):iv14-iv19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15923415

61. Bissell MJ, Barcellos-Hoff MH. The Influence of Extracellular Matrix on Gene Expression: Is Structure the Message? J Cell Sci [Internet]. 1987 [cited 2017 Sep 2];1987(Supplement 8). Available from: http://jcs.biologists.org/content/1987/Supplement_8/327

62. DeCosse JJ, Gossens CL, Kuzma JF, Unsworth BR. Breast cancer: induction of differentiation by embryonic tissue. Science [Internet]. 1973 Sep 14 [cited 2017 Sep 2];181(4104):1057–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4269415

63. Farber E. Pre-cancerous steps in carcinogenesis. Their physiological adaptive nature. Biochim Biophys Acta [Internet]. 1984 [cited 2017 Sep 2];738(4):171–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6394048

64. Barcellos-Hoff MH, Medina D. New highlights on stroma–epithelial interactions in breast cancer. Breast Cancer Res [Internet]. 2004 Feb 25 [cited 2017 Sep 2];7(1):33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15642180

65. Ben-Baruch A. The multifaceted roles of chemokines in malignancy. Cancer Metastasis Rev [Internet]. 2006 Dec 6 [cited 2017 May 22];25(3):357–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17016763

66. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, et al. Stromal gene expression predicts clinical outcome in breast cancer. Nat Med [Internet]. Nature Publishing Group; 2008 May 27 [cited 2017 May 22];14(5):518–27. Available from: http://www.nature.com/doifinder/10.1038/nm1764

67. Dalberg U, Markholst H, Hornum L. Both Gimap5 and the diabetogenic BBDP allele of Gimap5 induce apoptosis in T cells. Int Immunol [Internet]. 2007 Feb 20 [cited 2017 Sep 3];19(4):447–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17369194

68. Marchini C, Montani M, Konstantinidou G, Orrù R, Mannucci S, Ramadori G, et al. Mesenchymal/Stromal Gene Expression Signature Relates to Basal-Like Breast Cancers, Identifies Bone Metastasis and Predicts Resistance to Therapies. Shioda T, editor. PLoS One [Internet]. Public Library of Science; 2010 Nov 30 [cited 2017 May 22];5(11):e14131. Available from: http://dx.plos.org/10.1371/journal.pone.0014131

69. Tannock IF. The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. Br J Cancer [Internet]. Nature Publishing Group; 1968 Jun [cited 2017 May 22];22(2):258–73. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5660132

70. Tannock I. Cell kinetics and chemotherapy: a critical review. Cancer Treat Rep [Internet]. 1978 Aug [cited 2017 May 22];62(8):1117–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/356975

71. Trédan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. Journal of the National Cancer Institute. 2007. p. 1441–54.

72. Rice GC, Hoy C, Schimke RT. Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. Proc Natl Acad Sci U S A [Internet]. 1986 Aug [cited 2017 Sep 3];83(16):5978–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3461470

73. Wardman P. Electron transfer and oxidative stress as key factors in the design of drugs selectively active in hypoxia. Curr Med Chem [Internet]. 2001 Jun [cited 2017 Sep 3];8(7):739–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11375747

74. Dang C V, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci [Internet]. 1999 Feb [cited 2017 Sep 3];24(2):68–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10098401

75. Warburg O. On the Origin of Cancer Cells. Science (80-) [Internet]. 1956 [cited 2017 Sep 3];123(3191). Available from: http://science.sciencemag.org/content/123/3191/309

76. Tatum JL, Kelloff GJ, Gillies RJ, Arbeit JM, Brown JM, Chao KSC, et al. Hypoxia: Importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. Int J Radiat Biol [Internet]. 2006 Jan 3 [cited 2017 Sep 3];82(10):699–757. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17118889

77. Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. Semin Radiat Oncol [Internet]. 2004 Jul [cited 2017 Sep 3];14(3):198–206. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15254862

78. Tannock IF, Rotin D. Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res [Internet]. 1989 Aug 15 [cited 2017 May 22];49(16):4373–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2545340

79. Gerweck LE, Vijayappa S, Kozin S. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. Mol Cancer Ther [Internet]. 2006 May 1 [cited 2017 May 22];5(5):1275–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16731760

80. Lankelma J, Dekker H, Luque FR, Luykx S, Hoekman K, van der Valk P, et al. Doxorubicin gradients in human breast cancer. Clin Cancer Res [Internet]. 1999 Jul [cited 2017 May 22];5(7):1703–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10430072

81. Lee CM, Tannock IF. Inhibition of endosomal sequestration of basic anticancer drugs: influence on cytotoxicity and tissue penetration. Br J Cancer [Internet]. 2006 Mar 27 [cited 2017 May 22];94(6):863–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16495919

82. Tunggal JK, Melo T, Ballinger JR, Tannock IF. The influence of expression of P-glycoprotein on the penetration of anticancer drugs through multicellular layers. Int J Cancer. 2000;86(1):101–7.

83. Park CH, Bergsagel DE, McCulloch EA. Mouse myeloma tumor stem cells: a primary cell culture assay. J Natl Cancer Inst [Internet]. 1971 Feb [cited 2017 Sep 4];46(2):411–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5115909

84. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature [Internet]. Nature Publishing Group; 1994 Feb 17 [cited 2017 Sep 4];367(6464):645–8. Available from: http://www.nature.com/doifinder/10.1038/367645a0

85. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med [Internet]. 1998 Sep [cited 2017 Sep 4];4(9):1038–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9734397

86. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature [Internet]. 2007 Jan 4 [cited 2017 Sep

4];445(7123):106–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17122772

87. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective Identification of Tumorigenic Prostate Cancer Stem Cells. Cancer Res [Internet]. 2005 Dec 1 [cited 2017 Sep 4];65(23):10946–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16322242

88. Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H. Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun [Internet]. 2006 Dec 29 [cited 2017 Sep 4];351(4):820–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17097610

89. Izrailit J, Reedijk M. Developmental pathways in breast cancer and breast tumor-initiating cells: Therapeutic implications. Cancer Lett [Internet]. 2012 Apr 28 [cited 2017 Sep 4];317(2):115–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22123293

90. Neuzil J, Stantic M, Zobalova R, Chladova J, Wang X, Prochazka L, et al. Tumour-initiating cells vs. cancer “stem” cells and CD133: What’s in the name? Biochem Biophys Res Commun [Internet]. 2007 Apr 20 [cited 2017 Sep 4];355(4):855–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17307142

91. Liu C, Lu Y, Wang B, Zhang Y, Zhang R, Lu Y, et al. Clinical Implications of Stem Cell Gene Oct-4 Expression in Breast Cancer. Ann Surg [Internet]. 2011 Jun [cited 2017 Sep 4];253(6):1165–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21394007

92. Nakshatri H, Srour EF, Badve S. Breast cancer stem cells and intrinsic subtypes: controversies rage on. Curr Stem Cell Res Ther [Internet]. 2009 Jan [cited 2017 Sep 4];4(1):50–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19149630

93. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, et al. The Triple Negative Paradox: Primary Tumor Chemosensitivity of Breast Cancer Subtypes. Clin Cancer Res [Internet]. 2007 Apr 15 [cited 2017 Sep 4];13(8):2329–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17438091

94. Sorlie T, Perou CM, Fan C, Geisler S, Aas T, Nobel A, et al. Gene expression profiles do not consistently predict the clinical treatment response in locally advanced breast cancer. Mol Cancer Ther [Internet]. 2006 Nov 1 [cited 2017 Sep 4];5(11):2914–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17121939

95. Esserman LJ, Berry DA, Cheang MCU, Yau C, Perou CM, Carey L, et al. Chemotherapy response and recurrence-free survival in neoadjuvant breast cancer depends on biomarker profiles: results from the I-SPY 1 TRIAL (CALGB 150007/150012; ACRIN 6657). Breast Cancer Res Treat [Internet]. 2012 Apr 25 [cited 2017 Sep 4];132(3):1049–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22198468

96. Yoshimoto M, Takao S, Hirata M, Okamoto Y, Yamashita S, Kawaguchi Y, et al.

Metronomic oral combination chemotherapy with capecitabine and cyclophosphamide: a phase II study in patients with HER2-negative metastatic breast cancer. Cancer Chemother Pharmacol [Internet]. 2012 Aug 11 [cited 2017 Sep 4];70(2):331–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22526409

97. O'Shaughnessy J. Gemcitabine combination chemotherapy in metastatic breast cancer: phase II experience. Oncology (Williston Park) [Internet]. 2003 Dec [cited 2017 Sep 4];17(12 Suppl 14):15–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14768400

98. Akhtar N, Ahad A, Khar RK, Jaggi M, Aqil M, Iqbal Z, et al. The emerging role of P-glycoprotein inhibitors in drug delivery: a patent review. Expert Opin Ther Pat [Internet]. 2011 Apr 26 [cited 2017 May 21];21(4):561–76. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21413912

99. Bates SE, Chen C, Robey R, Kang M, Figg WD, Fojo T. Reversal of Multidrug Resistance: Lessons from Clinical Oncology. In John Wiley & Sons, Ltd; [cited 2017 May 21]. p. 83–102. Available from: http://doi.wiley.com/10.1002/0470846356.ch7

100. Plowe C V. Antimalarial drug resistance in Africa: strategies for monitoring and deterrence. Curr Top Microbiol Immunol [Internet]. 2005 [cited 2017 Sep 4];295:55–79. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16265887

101. Savarino A, Lucia MB, Giordano F, Cauda R. Risks and benefits of chloroquine use in anticancer strategies. Lancet Oncol [Internet]. 2006 Oct [cited 2017 Sep 4];7(10):792–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17012039

102. Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? Lancet Infect Dis [Internet]. 2003 Nov [cited 2017 Sep 4];3(11):722–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14592603

103. Sotelo J, Briceño E, López-González MA. Adding chloroquine to conventional treatment for glioblastoma multiforme: a randomized, double-blind, placebo-controlled trial. Ann Intern Med [Internet]. 2006 Mar 7 [cited 2017 Sep 4];144(5):337–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16520474

104. Kim EL, Wüstenberg R, Rübsam A, Schmitz-Salue C, Warnecke G, Bücker E-M, et al. Chloroquine activates the p53 pathway and induces apoptosis in human glioma cells. Neuro Oncol [Internet]. Oxford University Press; 2010 Apr [cited 2017 Sep 4];12(4):389–400. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20308316

105. Parissenti AM, Chapman J-AW, Kahn HJ, Guo B, Han L, O'Brien P, et al. Association of low tumor RNA integrity with response to chemotherapy in breast cancer patients. Breast Cancer Res Treat [Internet]. 2010 Jan 22 [cited 2017 Sep 4];119(2):347–56. Available

from: http://www.ncbi.nlm.nih.gov/pubmed/19771508

106. Gnant M, Steger GG, Shak S, America for TBCI of N, al. et. Fighting overtreatment in adjuvant breast cancer therapy. Lancet [Internet]. Elsevier; 2009 Dec 19 [cited 2017 Sep 4];374(9707):2029–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20004965

107. Albain KS, Barlow WE, Ravdin PM, Farrar WB, Burton G V, Ketchel SJ, et al. Adjuvant chemotherapy and timing of tamoxifen in postmenopausal patients with endocrine-responsive, node-positive breast cancer: a phase 3, open-label, randomised controlled trial. Lancet [Internet]. 2009 Dec 19 [cited 2017 Sep 4];374(9707):2055–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20004966

108. The Cells in Your Body [Internet]. American Association for the Advancement of Science. [cited 2016 Jul 5]. Available from: http://sciencenetlinks.com/student-teacher-sheets/cells-your-body/

109. Abbas AK, Lichtman AH, Pillai S. Innate Immunity. In: Cellular and Molecular Immunology. 7th ed. 2012. p. 58–9.

110. Nikaido H, Vaara M. Molecular Basis of Bacterial Outer Membrane Permeability. Microbiol Rev. 1985;49(1):1–32.

111. Dong H, Xiang Q, Gu Y, Wang Z, Paterson NG, Stansfeld PJ, et al. Structural basis for outer membrane lipopolysaccharide insertion. Nature [Internet]. Nature Research; 2014 Jun 18 [cited 2016 Oct 7];511(7507):52–6. Available from: http://www.nature.com/doifinder/10.1038/nature13464

112. Seydel U, Oikawa M, Fukase K, Kusumoto S, Brandenburg K. Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity. Eur J Biochem [Internet]. 2000 May [cited 2016 Sep 28];267(10):3032–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10806403

113. Qiao S, Luo Q, Zhao Y, Zhang XC, Huang Y. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. Nature [Internet]. 2014;511(7507):108–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24990751

114. Coats SR, Pham T-TT, Bainbridge BW, Reife RA, Darveau RP. MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize Escherichia coli lipopolysaccharide at the TLR4 signaling complex. J Immunol [Internet]. 2005 Oct 1 [cited 2017 May 26];175(7):4490–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16177092

115. Sweet MJ, Hume DA. Endotoxin signal transduction in macrophages. J Leukoc Biol. 1996;60(1):8–26.

116. Poltorak A. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4

Gene. Science (80-) [Internet]. American Association for the Advancement of Science; 1998 Dec 11 [cited 2015 Sep 17];282(5396):2085–8. Available from: http://science.sciencemag.org/content/282/5396/2085.abstract

117. Qureshi ST, Larivière L, Leveque G, Clermont S, Moore KJ, Gros P, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med [Internet]. 1999;189(4):615–25. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2192941&tool=pmcentrez&rendertype=abstract

118. Byrd CA, Bornmann W, Erdjument-Bromage H, Tempst P, Pavletich N, Rosen N, et al. Heat shock protein 90 mediates macrophage activation by Taxol and bacterial lipopolysaccharide. Proc Natl Acad Sci U S A [Internet]. 1999 May 11 [cited 2016 Jan 21];96(10):5645–50. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=21914&tool=pmcentrez&rendertype=abstract

119. Byrd-Leifer CA, Block EF, Takeda K, Akira S, Ding A. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. Eur J Immunol [Internet]. 2001 Aug [cited 2016 Jan 11];31(8):2448–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11500829

120. Chandrasoma P. Cardinal Clinical Signs. In: Concise Pathology. 3rd ed. New York, N.Y.: McGraw Hill; 2005.

121. Abbas AK, Lichtman AH, Pillai S. Properties and Overview of Immune Responses. In: Cellular and Molecular Immunology. 7th ed. 2012. p. 3.

122. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med [Internet]. 2002 Jan 7 [cited 2016 Sep 29];195(1):99–111. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11781369

123. Park JS, Svetkauskaite D, He Q, Kim J-Y, Strassheim D, Ishizaka A, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem [Internet]. 2004 Feb 27 [cited 2016 Sep 29];279(9):7370–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14660645

124. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81(1):1–5.

125. Müller S, Ronfani L, Bianchi ME. Regulated expression and subcellular localization of HMGB1, a chromatin protein with a cytokine function. J Intern Med [Internet]. 2004 Mar [cited 2016 Oct 6];255(3):332–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14871457

126. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and

infection. Annu Rev Immunol [Internet]. 2011 [cited 2016 Oct 6];29:139–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21219181

127. Yanai H, Ban T, Taniguchi T. Essential role of high-mobility group box proteins in nucleic acid-mediated innate immune responses. J Intern Med [Internet]. 2011 Oct [cited 2016 Oct 6];270(4):301–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21793952

128. Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, et al. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc Natl Acad Sci U S A [Internet]. 2010 Jun 29 [cited 2016 Oct 6];107(26):11942–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20547845

129. Stros M. HMGB proteins: interactions with DNA and chromatin. Biochim Biophys Acta [Internet]. [cited 2016 Oct 6];1799(1–2):101–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20123072

130. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature [Internet]. Nature Publishing Group; 2002 Jul 11 [cited 2016 Oct 6];418(6894):191–5. Available from: http://www.nature.com/doifinder/10.1038/nature00858

131. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell. 2011. p. 646–74.

132. Horwitz SB, Lothstein L, Manfredi JJ, Mellado W, Parness J, Roy SN, et al. Taxol: Mechanisms of Action and Resistance. Ann N Y Acad Sci [Internet]. Blackwell Publishing Ltd; 1986 Jun [cited 2016 Oct 4];466(1 Dynamic Aspec):733–44. Available from: http://doi.wiley.com/10.1111/j.1749-6632.1986.tb38455.x

133. Bogdan C, Ding A. Taxol, a microtubule-stabilizing antineoplastic agent, induces expression of tumor necrosis factor alpha and interleukin-1 in macrophages. J Leukoc Biol [Internet]. 1992 Jul 1 [cited 2016 Jan 18];52(1):119–21. Available from: http://www.jleukbio.org/content/52/1/119.abstract

134. Burkhart C a, Berman JW, Swindell CS, Horwitz SB. Relationship between the structure of taxol and other taxanes on induction of tumor necrosis factor-alpha gene expression and cytotoxicity. Cancer Res. 1994;54(22):5779–82.

135. Winefield RD, Entwistle RA, Foland TB, Lushington GH, Himes RH. Differences in paclitaxel and docetaxel interactions with tubulin detected by mutagenesis of yeast tubulin. ChemMedChem [Internet]. NIH Public Access; 2008 Dec [cited 2016 Oct 7];3(12):1844–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18988212

136. Yan HQ, Huang XB, Ke SZ, Jiang YN, Zhang YH, Wang YN, et al. Interleukin 6 augments lung cancer chemotherapeutic resistance via ataxia-telangiectasia mutated/NF-kappaB pathway activation. Cancer Sci [Internet]. 2014 Sep [cited 2016 Dec 14];105(9):1220–7.

Available from: http://www.ncbi.nlm.nih.gov/pubmed/24988892

137. Lev DC, Ruiz M, Mills L, McGary EC, Price JE, Bar-Eli M. Dacarbazine causes transcriptional up-regulation of interleukin 8 and vascular endothelial growth factor in melanoma cells: a possible escape mechanism from chemotherapy. Mol Cancer Ther. 2003;2(August):753–63.

138. Lev DC, Onn A, Melinkova VO, Miller C, Stone V, Ruiz M, et al. Exposure of Melanoma Cells to Dacarbazine Results in Enhanced Tumor Growth and Metastasis In Vivo. J Clin Oncol [Internet]. 2004 Apr 13 [cited 2016 Dec 12];22(11):2092–100. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15123733

139. Li G, Zhao L, Li W, Fan K, Qian W, Hou S, et al. Feedback activation of STAT3 mediates trastuzumab resistance via upregulation of MUC1 and MUC4 expression. Oncotarget [Internet]. 2014 Oct 4 [cited 2016 Dec 14];5(18):8317–29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25327561

140. Mahon KL, Lin H-M, Castillo L, Lee BY, Lee-Ng M, Chatfield MD, et al. Cytokine profiling of docetaxel-resistant castration-resistant prostate cancer. Br J Cancer [Internet]. 2015;112(8):1340–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25867259

141. Mahon FX, Belloc F, Lagarde V, Chollet C, Moreau-Gaudry F, Reiffers J, et al. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood. 2003;101(6):2368–73.

142. Lee LF, Haskill JS, Mukaida N, Matsushima K, Ting JP. Identification of tumor-specific paclitaxel (Taxol)-responsive regulatory elements in the interleukin-8 promoter. Mol Cell Biol [Internet]. 1997 Sep [cited 2016 Jan 21];17(9):5097–105. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=232360&tool=pmcentrez&rendertype=abstract

143. Lee LF, Schuerer-Maly CC, Lofquist AK, Van Haaften-Day C, Ting JPY, White CM, et al. Taxol-dependent transcriptional activation of IL-8 expression in a subset of human ovarian cancer. Cancer Res. 1996;56(6):1303–8.

144. Collins TS, Lee LF, Ting JP. Paclitaxel up-regulates interleukin-8 synthesis in human lung carcinoma through an NF-kappaB- and AP-1-dependent mechanism. Cancer Immunol Immunother [Internet]. 2000 May [cited 2016 Oct 13];49(2):78–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10823417

145. Wilson C, Purcell C, Seaton A, Oladipo O, Maxwell PJ, O'Sullivan JM, et al. Chemotherapy-induced CXC-chemokine/CXC-chemokine receptor signaling in metastatic prostate cancer cells confers resistance to oxaliplatin through potentiation of nuclear factor-kappaB transcription and evasion of apoptosis. J Pharmacol Exp Ther [Internet]. 2008 Dec [cited 2016 Oct 11];327(3):746–59. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/18780829

146. Acharyya S, Oskarsson T, Vanharanta S, Malladi S, Kim J, Morris PG, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. Cell [Internet]. 2012 Jul 6 [cited 2016 May 15];150(1):165–78. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3528019&tool=pmcentrez&rendertype=abstract

147. Levina V, Su Y, Nolen B, Liu X, Gordin Y, Lee M, et al. Chemotherapeutic drugs and human tumor cells cytokine network. Int J Cancer [Internet]. Wiley Subscription Services, Inc., A Wiley Company; 2008 Nov 1 [cited 2016 Dec 14];123(9):2031–40. Available from: http://doi.wiley.com/10.1002/ijc.23732

148. Sedgwick JD, Riminton DS, Cyster JG, K??rner H. Tumor necrosis factor: A master-regulator of leukocyte movement. Immunology Today. 2000. p. 110–3.

149. Edwardson DW, Boudreau J, Mapletoft J, Lanner C, Kovala AT, Parissenti AM. Inflammatory cytokine production in tumor cells upon chemotherapy drug exposure or upon selection for drug resistance. PLoS One [Internet]. 2017 [cited 2017 Sep 19];12(9):e0183662. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28915246

150. Berkova N, Page M. Addition of hTNFα potentiates cytotoxicity of taxol in human ovarian cancer lines. Anticancer Res [Internet]. International Institute of Anticancer Research; 1995 [cited 2016 Jan 19];15(3):863–6. Available from: http://cat.inist.fr/?aModele=afficheN&cpsidt=3614551

151. Li B, Vincent A, Cates J, Brantley-Sieders DM, Polk DB, Young PP. Low levels of tumor necrosis factor alpha increase tumor growth by inducing an endothelial phenotype of monocytes recruited to the tumor site. Cancer Res [Internet]. 2009 Jan 1 [cited 2016 Aug 17];69(1):338–48. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19118019

152. Ardestani S, Li B, Deskins DL, Wu H, Massion PP, Young PP. Membrane versus soluble isoforms of TNF-?? exert opposing effects on tumor growth and survival of tumor-associated myeloid cells. Cancer Res. 2013;73(13):3938–50.

153. Xie K. Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev. 2001;12(4):375–91.

154. Waugh DJJ, Wilson C. The interleukin-8 pathway in cancer. Clinical Cancer Research. 2008. p. 6735–41.

155. Tanaka T, Bai Z, Srinoulprasert Y, Yang B-G, Yang B, Hayasaka H, et al. Chemokines in tumor progression and metastasis. Cancer Sci [Internet]. 2005;96(6):317–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15958053

156. Merritt WM, Lin YG, Spannuth WA, Fletcher MS, Kamat AA, Han LY, et al. Effect of

interleukin-8 gene silencing with liposome-encapsulated small interfering RNA on ovarian cancer cell growth. J Natl Cancer Inst. 2008;100(5):359–72.

157. Wang Y, Qu Y, Niu XL, Sun WJ, Zhang XL, Li LZ. Autocrine production of interleukin-8 confers cisplatin and paclitaxel resistance in ovarian cancer cells. Cytokine [Internet]. 2011 Nov [cited 2016 Jan 8];56(2):365–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21742513

158. Walz A, Peveri P, Aschauer H, Baggiolini M. Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. Biochem Biophys Res Commun. 1987;149(2):755–61.

159. Schröder JM, Christophers E. Identification of C5ades arg and an anionic neutrophil-activating peptide (ANAP) in psoriatic scales. J Invest Dermatol [Internet]. 1986 Jul [cited 2016 Sep 15];87(1):53–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3014008

160. Matsushima K, Oppenheim JJ. Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF. Cytokine [Internet]. 1989 Nov [cited 2016 Sep 15];1(1):2–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2491503

161. Roebuck KA. Regulation of Interleukin-8 Gene Expression. J Interf Cytokine Res. 1999;19:429–38.

162. Xu L, Fidler IJ. Interleukin 8: an autocrine growth factor for human ovarian cancer. Oncol Res [Internet]. 2000;12(2):97–106. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11132928

163. Murdoch C, Monk PN, Finn a. Cxc chemokine receptor expression on human endothelial cells. Cytokine [Internet]. 1999;11(9):704–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10479407

164. Zhu YM, Webster SJ, Flower D, Woll PJ. Interleukin-8/CXCL8 is a growth factor for human lung cancer cells. Br J Cancer [Internet]. Nature Publishing Group; 2004 Nov 29 [cited 2016 Oct 13];91(11):1970–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15545974

165. Wang Y, Niu XL, Qu Y, Wu J, Zhu YQ, Sun WJ, et al. Autocrine production of interleukin-6 confers cisplatin and paclitaxel resistance in ovarian cancer cells. Cancer Lett [Internet]. 2010;295(1):110–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20236757

166. Duan Z, Feller AJ, Penson RT, Chabner BA, Seiden M V. Discovery of differentially expressed genes associated with paclitaxel resistance using cDNA array technology: Analysis of interleukin (IL) 6, IL-8, and monocyte chemotactic protein I in the paclitaxel-resistant phenotype. Clin Cancer Res. 1999;5(11):3445–53.

167. Kassim SK, El-Salahy EM, Fayed ST, Helal SA, Helal T, Azzam EE, et al. Vascular endothelial growth factor and interleukin-8 are associated with poor prognosis in epithelial ovarian cancer patients. Clin Biochem [Internet]. 2004 May [cited 2016 Sep 15];37(5):363–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15087251

168. Herrera CA, Xu L, Bucana CD, Silva el VG, Hess KR, Gershenson DM, et al. Expression of metastasis-related genes in human epithelial ovarian tumors. Int J Oncol [Internet]. 2002;20(1):5–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11743636

169. Zhang H, Wu H, Guan J, Wang L, Ren X, Shi X, et al. Paracrine SDF-1α signaling mediates the effects of PSCs on GEM chemoresistance through an IL-6 autocrine loop in pancreatic cancer cells. Oncotarget [Internet]. 2015 Feb 7 [cited 2016 Dec 14];6(5):3085–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25609203

170. Korkaya H, Kim G Il, Davis A, Malik F, Henry NL, Ithimakin S, et al. Activation of an IL6 Inflammatory Loop Mediates Trastuzumab Resistance in HER2+ Breast Cancer by Expanding the Cancer Stem Cell Population. Mol Cell. 2012;47(4):570–84.

171. Yang Z, Guo L, Liu D, Sun L, Chen H, Deng Q, et al. Acquisition of resistance to trastuzumab in gastric cancer cells is associated with activation of IL-6/STAT3/Jagged-1/Notch positive feedback loop. Oncotarget [Internet]. 2015 Mar 6 [cited 2016 Dec 14];6(7):5072–87. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25669984

172. Frassanito MA, Cusmai A, Iodice G, Dammacco F. Autocrine interleukin-6 production and highly malignant multiple myeloma: Relation with resistance to drug-induced apoptosis. Blood. 2001;97(2):483–9.

173. Duan S, Tsai Y, Keng P, Chen Y, Ok Lee S, Chen Y. IL-6 signaling contributes to cisplatin resistance in non-small cell lung cancer via the up-regulation of anti-apoptotic and DNA repair associated molecules. Oncotarget [Internet]. 2015 Sep 28 [cited 2016 Dec 14];6(29):27651–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26313152

174. Suh Y-A, Jo S-Y, Lee H-Y, Lee C. Inhibition of IL-6/STAT3 axis and targeting Axl and Tyro3 receptor tyrosine kinases by apigenin circumvent taxol resistance in ovarian cancer cells. Int J Oncol [Internet]. 2014 Dec 23 [cited 2016 Dec 14];46(3):1405–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25544427

175. Rodriguez-Barrueco R, Yu J, Saucedo-Cuevas LP, Olivan M, Llobet-Navas D, Putcha P, et al. Inhibition of the autocrine IL-6–JAK2–STAT3–calprotectin axis as targeted therapy for HR $^{-}$ /HER2 $^{+}$ breast cancers. Genes Dev [Internet]. 2015 Aug 1 [cited 2016 Dec 14];29(15):1631–48. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26227964

176. Conze D, Weiss L, Regen PS, Bhushan A, Weaver D, Johnson P, et al. Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. Cancer Res. 2001;61(24):8851–8.

177. Wang Y, Niu XL, Guo XQ, Yang J, Li L, Qu Y, et al. IL6 induces TAM resistance via kinase-specific phosphorylation of ERα in OVCA cells. J Mol Endocrinol. 2015;54(3):351–61.

178. Hartman ZC, Poage GM, Den Hollander P, Tsimelzon A, Hill J, Panupinthu N, et al. Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. Cancer Res. 2013;73(11):3470–80.

179. Ghandadi M, Sahebkar A. Interleukin-6: A Critical Cytokine in Cancer Multidrug Resistance. Curr Pharm Des [Internet]. 2016 [cited 2016 Dec 14];22(5):518–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26601970

180. Kumari N, Dwarakanath BS, Das A, Bhatt AN. Role of interleukin-6 in cancer progression and therapeutic resistance. Tumor Biol [Internet]. 2016 Sep 3 [cited 2016 Dec 14];37(9):11553–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27260630

181. De Filippo K, Dudeck A, Hasenberg M, Nye E, Van Rooijen N, Hartmann K, et al. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood. 2013;121(24):4930–7.

182. Bolitho C, Hahn MA, Baxter RC, Marsh DJ. The chemokine CXCL1 induces proliferation in epithelial ovarian cancer cells by transactivation of the epidermal growth factor receptor. Endocr Relat Cancer. 2010;17(4):929–40.

183. Dhawan P, Richmond A. Role of CXCL1 in tumorigenesis of melanoma. J Leukoc Biol. 2002;72(1):9–18.

184. Owen JD, Strieter R, Burdick M, Haghnegahdar H, Nanney L, Shattuck-Brandt R, et al. Enhanced tumor-forming capacity for immortalized melanocytes expressing melanoma growth stimulatory activity/growth-regulated cytokine beta and gamma proteins. Int J cancer [Internet]. 1997 Sep 26 [cited 2016 Oct 18];73(1):94–103. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9334815

185. Lee LF, Hellendall RP, Wang Y, Haskill JS, Mukaida N, Matsushima K, et al. IL-8 reduced tumorigenicity of human ovarian cancer in vivo due to neutrophil infiltration. J Immunol [Internet]. American Association of Immunologists; 2000 Mar 1 [cited 2016 Sep 15];164(5):2769–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10679119

186. Huang S, Mills L, Mian B, Tellez C, McCarty M, Yang X-D, et al. Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma. Am J Pathol [Internet]. 2002 Jul [cited 2016 Sep 27];161(1):125–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12107097

187. Penson RT, Kronish K, Duan Z, Feller AJ, Stark P, Cook SE, et al. Cytokines IL-1beta, IL-2, IL-6, IL-8, MCP-1, GM-CSF and TNFalpha in patients with epithelial ovarian cancer and their relationship to treatment with paclitaxel. Int J Gynecol Cancer [Internet]. 2000 Jan [cited 2016 Oct 5];10(1):33–41. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/11240649

188. Pusztai L, Mendoza TR, Reuben JM, Martinez MM, Willey JS, Lara J, et al. Changes in plasma levels of inflammatory cytokines in response to paclitaxel chemotherapy. Cytokine. 2004;25(3):94–102.

189. Hu G, Chong RA, Yang Q, Wei Y, Blanco MA, Li F, et al. MTDH Activation by 8q22 Genomic Gain Promotes Chemoresistance and Metastasis of Poor-Prognosis Breast Cancer. Cancer Cell. 2009;15(1):9–20.

190. Morris PG, McArthur HL, Hudis CA. Therapeutic options for metastatic breast cancer. Expert Opin Pharmacother [Internet]. 2009;10(6):967–81. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-67649592084&partnerID=tZOtx3y1

191. Modur V, Zimmerman GA, Prescott SM, McIntyre TM. Endothelial cell inflammatory responses to tumor necrosis factor alpha. Ceramide-dependent and -independent mitogen-activated protein kinase cascades. J Biol Chem [Internet]. American Society for Biochemistry and Molecular Biology; 1996 May 31 [cited 2016 Oct 12];271(22):13094–102. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8662702

192. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature [Internet]. 1997 Feb 20 [cited 2015 Sep 5];385(6618):729–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9034190

193. Shedden K, Taylor JMG, Enkemann SA, Tsao M-S, Yeatman TJ, Gerald WL, et al. Gene expression-based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. Nat Med [Internet]. 2008;14(8):822–7. Available from: http://www.nature.com.ezp-prod1.hul.harvard.edu/nm/journal/v14/n8/full/nm.1790.html

194. Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunological Reviews. 2007. p. 47–59.

195. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med [Internet]. 2007;13(9):1050–9. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17704786%5Cnhttp://www.nature.com/nm/journal/v13/n9/pdf/nm1622.pdf

196. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini J-L, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med. 2007;13(1):54–61.

197. Obeid M, Panaretakis T, Tesniere A, Joza N, Tufi R, Apetoh L, et al. Leveraging the immune system during chemotherapy: Moving calreticulin to the cell surface converts apoptotic death from “silent” to immunogenic. Cancer Research. 2007. p. 7941–4.

198. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity [Internet]. 2013 Apr 18 [cited 2016 Oct 20];38(4):729–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23562161

199. Kepp O, Tesniere A, Schlemmer F, Michaud M, Senovilla L, Zitvogel L, et al. Immunogenic cell death modalities and their impact on cancer treatment. Apoptosis [Internet]. 2009 Apr [cited 2016 Oct 6];14(4):364–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19145485

200. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Mouse Toll-like Receptor 4·MD-2 Complex Mediates Lipopolysaccharide-mimetic Signal Transduction by Taxol. J Biol Chem [Internet]. 2000;275(4):2251–4. Available from: http://www.jbc.org/content/275/4/2251.short%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/10644670

201. Parker, M. M., Parrillo JE. Septic Shock. Hemodynamics and pathogenesis. JAMA. 1983;(250):3324–7.

202. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, et al. Shock and tissue injury induced by recombinant human cachectin. Science [Internet]. 1986 Oct 24 [cited 2016 Jan 13];234(4775):470–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3764421

203. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like Receptor-4 Mediates Lipopolysaccharide-induced Signal Transduction. J Biol Chem [Internet]. 1999 Apr 16 [cited 2016 Jan 10];274(16):10689–92. Available from: http://www.jbc.org/content/274/16/10689.full

204. Schletter J, Heine H, Ulmer AJ, Rietschel ET. Molecular mechanisms of endotoxin activity. Arch Microbiol [Internet]. 1995 Dec [cited 2016 Jan 13];164(6):383–9. Available from: http://link.springer.com/10.1007/BF02529735

205. Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature [Internet]. Macmillan Magazines Ltd.; 1998 Sep 17 [cited 2015 Dec 8];395(6699):284–8. Available from: http://dx.doi.org/10.1038/26239

206. Vitiello M, D’Isanto M, Galdiero M, Raieta K, Tortora A, Rotondo P, et al. Interleukin-8 production by THP-1 cells stimulated by Salmonella enterica serovar Typhimurium porins is mediated by AP-1, NF-kappaB and MAPK pathways. Cytokine. 2004;27(1):15–24.

207. Szajnik M, Szczepanski MJ, Czystowska M, Elishaev E, Mandapathil M, Nowak-Markwitz E, et al. TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. Oncogene [Internet]. 2009 Oct 12 [cited 2016 Jan 8];28(49):4353–63. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2794996&tool=pmcentrez&rendertype=abstract

208. Kelly MG, Alvero AB, Chen R, Silasi D-A, Abrahams VM, Chan S, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res [Internet]. 2006 Apr 1 [cited 2016 Jan 8];66(7):3859–68. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16585214

209. Wang AC, Ma YB, Wu FX, Ma ZF, Liu NF, Gao R, et al. TLR4 induces tumor growth and inhibits paclitaxel activity in MyD88-positive human ovarian carcinoma in vitro. Oncol Lett. 2014;7(3):871–7.

210. Wu Z, Wang S, Wu I, Mata M, Fink DJ. Activation of TLR-4 to produce tumour necrosis factor-α in neuropathic pain caused by paclitaxel. Eur J Pain [Internet]. 2015 Aug [cited 2016 Feb 4];19(7):889–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25388329

211. Armstrong SR, Narendrula R, Guo B, Parissenti AM, McCallum KL, Cull S, et al. Distinct genetic alterations occur in ovarian tumor cells selected for combined resistance to carboplatin and docetaxel. J Ovarian Res [Internet]. BioMed Central; 2012 Nov 30 [cited 2016 Jan 8];5(1):40. Available from: http://ovarianresearch.biomedcentral.com/articles/10.1186/1757-2215-5-40

212. Reife RA, Darveau RP, Coats SR, Pham T-TT, Bainbridge BW. MD-2 Mediates the Ability of Tetra-Acylated MD-2 Mediates the Ability of Tetra-Acylated and Penta-Acylated Lipopolysaccharides to Antagonize Escherichia coli Lipopolysaccharide at the TLR4 Signaling Complex. J Immunol Ref [Internet]. 2005 [cited 2016 Sep 8];175:4490–8. Available from: http://www.jimmunol.org/content/175/7/4490

213. Golenbock DT, Hampton RY, Qureshi N, Takayama K, Raetz CR. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. J Biol Chem [Internet]. 1991 Oct 15 [cited 2016 Sep 8];266(29):19490–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1918061

214. Visintin A, Halmen K, Latz E, Monks B, Golenbock D. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. Open Access Artic [Internet]. 2005 [cited 2016 Sep 8]; Available from: http://escholarship.umassmed.edu/oapubs/1092

215. Saitoh S, Akashi S, Yamada T, Tanimura N, Kobayashi M, Konno K, et al. Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-

MD-2 and ligand-induced TLR4 oligomerization. Int Immunol [Internet]. 2004 Jul [cited 2016 Sep 8];16(7):961–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15184344

216. Matsunaga N, Tsuchimori N, Matsumoto T, Ii M. TAK-242 (Resatorvid), a Small-Molecule Inhibitor of Toll-Like Receptor (TLR) 4 Signaling, Binds Selectively to TLR4 and Interferes with Interactions between TLR4 and Its Adaptor Molecules. Mol Pharmacol [Internet]. 2011 Jan 1 [cited 2017 Jun 27];79(1):34–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20881006

217. Stanley AC, Lacy P. Pathways for cytokine secretion. Physiology (Bethesda) [Internet]. 2010 Aug 1 [cited 2015 Nov 30];25(4):218–29. Available from: http://physiologyonline.physiology.org/content/25/4/218

218. Raabe T, Bukrinsky M, Currie RA. Relative contribution of transcription and translation to the induction of tumor necrosis factor-alpha by lipopolysaccharide. J Biol Chem [Internet]. 1998 Jan 9 [cited 2016 Jan 15];273(2):974–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9422758

219. Rasmussen HS, McCann PP. Matrix metalloproteinase inhibition as a novel anticancer strategy: A review with special focus on Batimastat and Marimastat. Pharmacology and Therapeutics. 1997. p. 69–75.

220. Reed K, Hembruff SL, Laberge ML, Villeneuve DJ, Côté GB, Parissenti AM. Hypermethylation of the ABCB1 downstream gene promoter accompanies ABCB1 gene amplification and increased expression in docetaxel-resistant MCF-7 breast tumor cells. Epigenetics [Internet]. 2008;3(5):270–80. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=19001875&retmode=ref&cmd=prlinks%5Cnpapers2://publication/uuid/0AFF11CD-E028-4015-9F4F-18C8AA4DA6D2

221. Shirakawa K, Takara K, Tanigawara Y, Aoyama N, Kasuga M, Komada F, et al. Interaction of docetaxel (“Taxotere”) with human P-glycoprotein. Jpn J Cancer Res [Internet]. 1999;90(12):1380–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10665657

222. Nobili S, Landini I, Giglioni B, Mini E. Pharmacological strategies for overcoming multidrug resistance. Curr Drug Targets [Internet]. 2006;7(7):861–79. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16842217

223. Manna SK, Ramesh GT. Interleukin-8 induces nuclear transcription factor-kappaB through a TRAF6-dependent pathway. J Biol Chem [Internet]. 2005 Feb 25 [cited 2016 Jan 27];280(8):7010–21. Available from: http://www.jbc.org/content/280/8/7010.abstract

224. Jeyaseelan S, Cai S, Batra S, Lira SA, Kolls JK. CXCL1 Regulates Pulmonary Host Defense CXCL1 Regulates Pulmonary Host Defense to Klebsiella Infection via CXCL2, CXCL5, NF-kB,

and MAPKs. J Immunol J Immunol [Internet]. 2010 [cited 2016 Aug 3];185(185):6214–25. Available from: http://www.jimmunol.org/content/185/10/6214

225. Zhang Y, Wang Y, Yuan J, Qin W, Liu F, Wang F, et al. Toll-like receptor 4 ligation confers chemoresistance to docetaxel on PC-3 human prostate cancer cells. Cell Biol Toxicol. 2012;28(4):269–77.

226. Baker SD, Zhao M, Lee CKK, Verweij J, Zabelina Y, Brahmer JR, et al. Comparative pharmacokinetics of weekly and every-three-weeks docetaxel. Clin Cancer Res [Internet]. Clinical Cancer Research; 2004 Mar 15 [cited 2016 Jul 19];10(6):1976–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15041715

227. Zimmer SM, Liu J, Clayton JL, Stephens DS, Snyder JP. Paclitaxel binding to human and murine MD-2. J Biol Chem. 2008;283(41):27916–26.

228. Resman N, Gradišar H, Vašl J, Keber MM, Pristovšek P, Jerala R. Taxanes inhibit human TLR4 signaling by binding to MD-2. FEBS Lett [Internet]. 2008;582(28):3929–34. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0014579308008697

229. Kawai T, Takeuchi O, Fujita T, Inoue J -i., Muhlradt PF, Sato S, et al. Lipopolysaccharide Stimulates the MyD88-Independent Pathway and Results in Activation of IFN-Regulatory Factor 3 and the Expression of a Subset of Lipopolysaccharide-Inducible Genes. J Immunol [Internet]. American Association of Immunologists; 2001 Nov 15 [cited 2016 Jan 14];167(10):5887–94. Available from: http://www.jimmunol.org/content/167/10/5887.full

230. Shurety W, Merino-Trigo A, Brown D, Hume DA, Stow JL. Localization and post-Golgi trafficking of tumor necrosis factor-alpha in macrophages. J Interferon Cytokine Res [Internet]. 2000 Apr [cited 2016 Jan 15];20(4):427–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10805378

231. Armstrong L, Godinho SIH, Uppington KM, Whittington HA, Millar AB. Contribution of TNF-α converting enzyme and proteinase-3 to TNF-α processing in human alveolar macrophages. Am J Respir Cell Mol Biol. 2006;34(2):219–25.

232. Biedler JL, Riehm H. Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies. Cancer Res. 1970;30(4):1174–84.

233. Erba E, Bergamaschi D, Bassano L, Ronzoni S, Di Liberti G, Muradore I, et al. Isolation and characterization of an IGROV-1 human ovarian cancer cell line made resistant to Ecteinascidin-743 (ET-743). Br J Cancer [Internet]. 2000;82(10):1732–9. Available from: www.ncbi.nlm.nih.gov/pubmed/10817511

234. Goldstein LJ, Galski H, Fojo a, Willingham M, Lai SL, Gazdar a, et al. Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst. 1989;81:116–24.

235. Miller TP, Grogan TM, Dalton WS, Spier CM, Scheper RJ, Salmon SE. P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high-dose verapamil. J Clin Oncol [Internet]. 1991 Jan [cited 2017 May 20];9(1):17–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1670642

236. Taylor CW, Dalton WS, Mosley K, Dorr RT, Salmon SE. Combination chemotherapy with cyclophosphamide, vincristine, adriamycin, and dexamethasone (CVAD) plus oral quinine and verapamil in patients with advanced breast cancer. Breast Cancer Res Treat [Internet]. Kluwer Academic Publishers; 1997 Jan [cited 2017 May 20];42(1):7–14. Available from: http://link.springer.com/10.1023/A:1005716214718

237. Trock BJ, Leonessa F, Clarke R. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. J Natl Cancer Inst [Internet]. 1997;89(13):917–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9214671

238. Nobili S, Napoli C, Puccini B, Landini I, Perrone G, Brugia M, et al. Identification of pharmacogenomic markers of clinical efficacy in a dose-dense therapy regimen (R-CHOP14) in diffuse large B-cell lymphoma. Leuk Lymphoma [Internet]. 2014;(August 2013):1–8. Available from: http://informahealthcare.com/doi/abs/10.3109/10428194.2013.866665%5Cnpapers2://publication/doi/10.3109/10428194.2013.866665%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/24289107

239. Sharma B, Nawandar DM, Nannuru KC, Varney ML, Singh RK. Targeting CXCR2 enhances chemotherapeutic response, inhibits mammary tumor growth, angiogenesis, and lung metastasis. Mol Cancer Ther [Internet]. 2013 May [cited 2016 Jul 28];12(5):799–808. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23468530

240. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro Oncol [Internet]. 2005;7(2):122–33. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1871893&tool=pmcentrez&rendertype=abstract

241. Al-Bataineh MM, van der MD, Schultz BD, Gehring R. Tumor necrosis factor alpha increases P-glycoprotein expression in a BME-UV in vitro model of mammary epithelial cells. BiopharmDrug Dispos. 2010;31(1099–081X (Electronic)):506–15.

242. Yu C, Kastin AJ, Tu H, Waters S, Pan W. TNF activates P-glycoprotein in cerebral microvascular endothelial cells. Cell Physiol Biochem. 2007;20(6):853–8.

243. Bauer B, Hartz AMS, Miller DS. Tumor necrosis factor alpha and endothelin-1 increase P-glycoprotein expression and transport activity at the blood-brain barrier. Mol Pharmacol [Internet]. 2007;71(3):667–75. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/17132686

244. Masereeuw R, Heemskerk S, Peters JGP, Louisse J, Sagar S, Russel FGM. Regulation of P-glycoprotein in renal proximal tubule epithelial cells by LPS and TNF-?? J Biomed Biotechnol. 2010;2010.

245. Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, et al. Tumor necrosis factor-?? is a potent endogenous mutagen that promotes cellular transformation. Cancer Res. 2006;66(24):11565–70.

246. Rajput S, Volk-Draper LD, Ran S. TLR4 is a novel determinant of the response to paclitaxel in breast cancer. Mol Cancer Ther [Internet]. 2013;12(8):1676–87. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3742631&tool=pmcentrez&rendertype=abstract

247. Ran S. The role of TLR4 in chemotherapy-driven metastasis. Cancer Research. 2015. p. 2405–10.

248. Buoncervello M, Borghi P, Romagnoli G, Spadaro F, Belardelli F, Toschi E, et al. Apicidin and docetaxel combination treatment drives CTCFL expression and HMGB1 release acting as potential antitumor immune response inducers in metastatic breast cancer cells. Neoplasia [Internet]. 2012;14(9):855–67. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3459281&tool=pmcentrez&rendertype=abstract

249. Karikó K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem [Internet]. 2004;279(13):12542–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14729660

250. Ohashi K, Burkart V, Flohe S, Kolb H. Heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. JImmunol [Internet]. 2000;164(2):558–61. Available from: http://www.jimmunol.org/cgi/reprint/164/2/558.pdf

251. Ma Y, Temkin V, Liu H, Pope RM. NF-kappaB protects macrophages from lipopolysaccharide-induced cell death: the role of caspase 8 and receptor-interacting protein. J Biol Chem [Internet]. 2005 Dec 23 [cited 2016 Jan 28];280(51):41827–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16246838

252. Fried S, Tosun S, Troost G, Keil S, Zaenker KS, Dittmar T. Lipopolysaccharide (LPS) Promotes Apoptosis in Human Breast Epithelial × Breast Cancer Hybrids, but Not in Parental Cells. Lai H-C, editor. PLoS One [Internet]. 2016 Feb 10 [cited 2016 Oct 19];11(2):e0148438. Available from: http://dx.plos.org/10.1371/journal.pone.0148438

253. Núñez Miguel R, Wong J, Westoll JF, Brooks HJ, O'Neill LAJ, Gay NJ, et al. A Dimer of the Toll-Like Receptor 4 Cytoplasmic Domain Provides a Specific Scaffold for the Recruitment of Signalling Adaptor Proteins. Klefstrom J, editor. PLoS One [Internet]. 2007 Aug 29

[cited 2017 Jun 1];2(8):e788. Available from: http://dx.plos.org/10.1371/journal.pone.0000788

254. Lo H, Lai T, Li C, Wu W. TNF-α induces CXCL1 chemokine expression and release in human vascular endothelial cells in vitro via two distinct signaling pathways. Acta Pharmacol Sin [Internet]. 2014 Mar [cited 2016 Oct 19];35(3):339–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24487964

255. Beroukhim R, Mermel C, Porter D. The landscape of somatic copy-number alteration across human cancers. Nature [Internet]. 2010;463(February):899–905. Available from: http://www.nature.com/nature/journal/v463/n7283/abs/nature08822.html

256. Bièche I, Chavey C, Andrieu C, Busson M, Vacher S, Le Corre L, et al. CXC chemokines located in the 4q21 region are up-regulated in breast cancer. Endocr Relat Cancer. 2007;14(4):1039–52.

257. Dowling JK, Mansell A. Toll-like receptors: the swiss army knife of immunity and vaccine development. Clin Transl Immunol [Internet]. Nature Publishing Group; 2016 May 20 [cited 2017 May 23];5(5):e85. Available from: http://www.nature.com/doifinder/10.1038/cti.2016.22

258. Johnson AG, Gaines S, Landy M. Studies on the O antigen of Salmonella typhosa. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. J Exp Med [Internet]. 1956 Feb 1 [cited 2017 May 23];103(2):225–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13286429

259. Moore A, McCarthy L, Mills KH. The adjuvant combination monophosphoryl lipid A and QS21 switches T cell responses induced with a soluble recombinant HIV protein from Th2 to Th1. Vaccine [Internet]. 1999 Jun 4 [cited 2017 May 23];17(20–21):2517–27. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10418898

260. Baldridge JR, Cluff CW, Evans JT, Lacy MJ, Stephens JR, Brookshire VG, et al. Immunostimulatory activity of aminoalkyl glucosaminide 4-phosphates (AGPs): induction of protective innate immune responses by RC-524 and RC-529. J Endotoxin Res [Internet]. 2002 Dec 1 [cited 2017 May 23];8(6):453–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12697089

261. Stanberry LR, Spruance SL, Cunningham AL, Bernstein DI, Mindel A, Sacks S, et al. Glycoprotein-D–Adjuvant Vaccine to Prevent Genital Herpes. N Engl J Med [Internet]. Massachusetts Medical Society ; 2002 Nov 21 [cited 2017 May 23];347(21):1652–61. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa011915

262. Sokal EM, Hoppenbrouwers K, Vandermeulen C, Moutschen M, Léonard P, Moreels A, et al. Recombinant gp350 Vaccine for Infectious Mononucleosis: A Phase 2, Randomized, Double-Blind, Placebo-Controlled Trial to Evaluate the Safety, Immunogenicity, and

Efficacy of an Epstein-Barr Virus Vaccine in Healthy Young Adults. J Infect Dis [Internet]. 2007 Dec 15 [cited 2017 May 23];196(12):1749–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18190254

263. Penny MA, Verity R, Bever CA, Sauboin C, Galactionova K, Flasche S, et al. Public health impact and cost-effectiveness of the RTS,S/AS01 malaria vaccine: a systematic comparison of predictions from four mathematical models. Lancet [Internet]. 2016 Jan [cited 2017 May 23];387(10016):367–75. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0140673615007254

264. Bhatla N, Suri V, Basu P, Shastri S, Datta SK, Bi D, et al. Immunogenicity and safety of human papillomavirus-16/18 AS04-adjuvanted cervical cancer vaccine in healthy Indian women. J Obstet Gynaecol Res [Internet]. Blackwell Publishing Asia; 2010 Feb [cited 2017 May 23];36(1):123–32. Available from: http://doi.wiley.com/10.1111/j.1447-0756.2009.01167.x

265. Mitchell MS, Harel W, Kempf RA, Hu E, Kan-Mitchell J, Boswell WD, et al. Active-specific immunotherapy for melanoma. J Clin Oncol [Internet]. 1990 May [cited 2017 May 23];8(5):856–69. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2139701

266. Wright S, Ramos R, Tobias P, Ulevitch R, Mathison J. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science (80-) [Internet]. 1990;249(4975):1431–3. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1698311

Appendix - Appended figures, optimization experiments and unpublished data

Several experiments were conducted and preliminary data was obtained in order to supplement particular findings or optimize treatment conditions for use in certain experiments from which findings were presented in the results section of the thesis. The following is an assortment of this data, grouped into sections based on the method used, and presented as figures with explanatory text and commentary.

Reverse-transcription followed by qPCR (figures A1-A4)

Figure A1 presents data that was generated in order to assess whether TNF-α levels were driving cell signalling involved in increased extrusion of docetaxel from drug-resistant cells. As explained in the results section, increased expression of the cell membrane protein, P-gp, was found to be mediating the major mode of drug resistance, which was extrusion of docetaxel from the cell, in the MCF-7_{TXT10} and $_{A2780DXL12}$ cell lines (figure 3.7 and 3.8). Thus, we measured the relative levels of P-gp after treatment with exogenous TNF-α in drug-naive MCF-7 to see if TNF-α could drive an increase in P-gp expression at the protein level. The experiment would have benefited from including a treatment of MCF-7_{TXT10} with exogenous TNF-α as well, given that drug-naive MCF-7 may have undergone other changes in the acquisition of drug resistance that would permit TNF-α signalling to regulate P-gp expression.

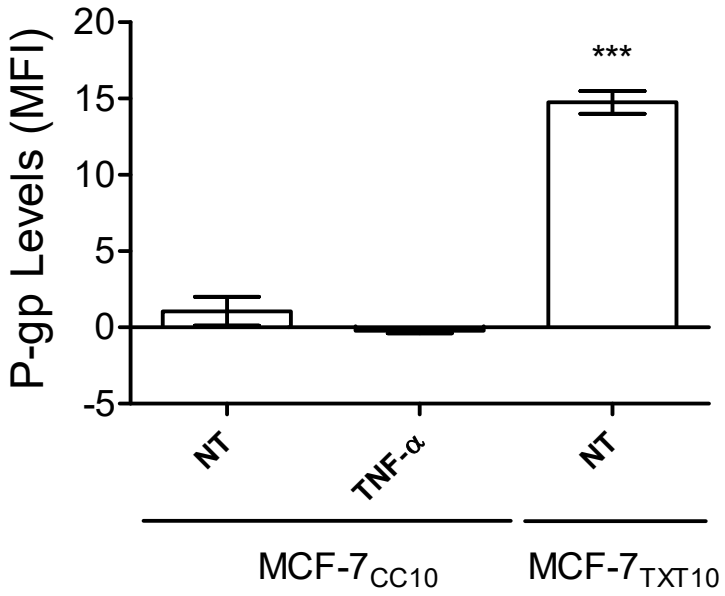

Figure A1 - Levels of P-gp protein expression in breast tumour cells with or without supplementation with exogenous recombinant TNF-α. The medium of MCF-7CC10 cells was supplemented with 10 ng/ml TNF- twice over 96 hours (second treatment at 48 hours), after which cell surface P-gp protein expression was assessed in treated and untreated cells by flow cytometry. This was then compared to P-gp expression in untreated MCF-7TXT10 cells using the same approach. An ANOVA with Tukey post-test was then used to assess the significance of differences in P-gp expression among the samples (***p=0.0002).

Figure 3.3D presents data from a qPCR experiment showing that increased TNF-α mRNA transcription occurs after exposure to 2.5 nM docetaxel for 36 hours, however, the 36-hour time point had to be first decided upon through experimentation and the generation of figure A2 (below).

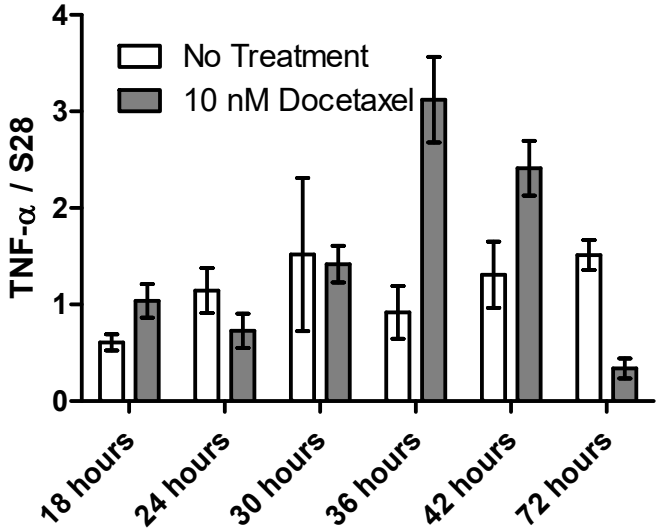

Figure A2 - TNF-α transcript levels in response to docetaxel. Cells were plated for 18, 24, 30, 36, 42, and 72 hours in the presence or absence of 10 nM docetaxel. cDNA was generated by reverse transcription of RNA extracts from MCF-7 cells, which was then assessed for TNF-α and S28 (control gene) transcript by quantitative PCR. Each treatment was performed in duplicate and each of the replicates were plated in triplicate on a 96-well qPCR plate.

Given the data presented in figure 3.4, where it was found that CXCL8 levels in the media increased slightly with the treatment of docetaxel, it was of interest to determine whether similar increases would be observed in CXCL8 mRNA transcript. Figure A3, presented below, does not demonstrate increased CXCL8 mRNA transcript caused by docetaxel, however it does not necessarily contradiction with our protein data for CXCL8, presented in figure 3.4, because it is quite possible that the time points used here were not optimal, nor the concentration of drug used.

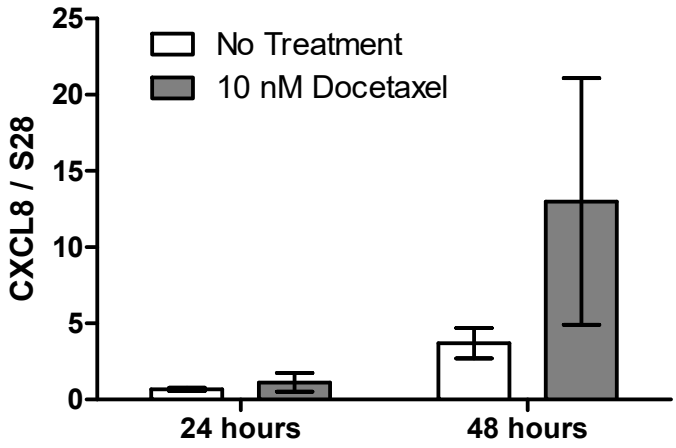

Figure A3 - CXCL8 transcript levels in response to docetaxel. Cells were plated for 24 and 48 hours in the presence or absence of 10 nM docetaxel. cDNA was generated by reverse transcription of RNA extracts from MCF-7 cells, which was then assessed for CXCL8 and S28 (control gene) transcript by quantitative PCR. Each treatment was performed in duplicate and each of the replicates were plated in triplicate on a 96-well qPCR plate.

Figure 3.9 from the results section presents data that suggests an increase in the basal release of TNF-α and CXCL1 during the acquisition of drug resistance for tumour cell lines of breast (MCF-7) ovarian (A2780) origin. Previously, our lab had shown that TNF-α mRNA transcript is also increased in the drug-resistant MCF-7_{TXT10} cell line relative to the drug-naive control cell line, thus with goal of determining whether a similar trend occurs with CXCL1, at the level of the transcript, the appropriate experimentation was performed to generate figure A4 (below). This data suggests an increase in basal CXCL1 mRNA transcription in MCF-7_{TXT10}. relative to MCF-7 co-cultured control cells.

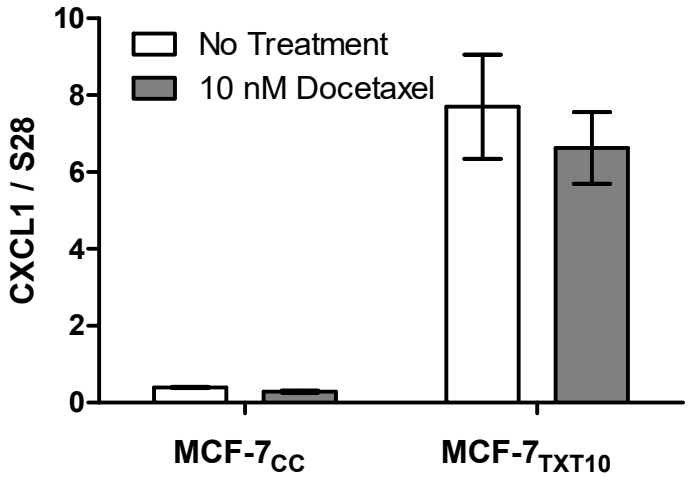

Figure A4 - CXCL1 transcript levels in docetaxel-sensitive and docetaxel-resistant cells. Cells were plated for 36 hours in the presence or absence of 10 nM docetaxel. cDNA was generated by reverse transcription of RNA extracts from both drug-sensitive (MCF-7_{cc}) and drug-resistant (MCF-7_{TXT10}) cells, which was then assessed for CXCL1 and S28 (control gene) transcript by quantitative PCR. Each treatment was performed in duplicate and each of the replicates were plated in triplicate on a 96-well qPCR plate.

Data presented in figure 3.9 from the results section shows the basal level of release for inflammatory cytokines TNF-α, CXCL1, and CXCL8, however, the decision to study these three cytokines was partly based on data generated using multiplex (Bioplex) instrumentation to measure levels of multiple cytokines in a given sample. The original panel of cytokines tested included RANTES (CCL5), MCP-1 (CCL2), CXCL1, CXCL8, TNF-α, IL-1β, and IL-6. IL-1β levels were below the level of detection for all cell lines and conditions originally tested, and thus experimentation concerning this cytokine did not continue. Likewise, experiments concerning IL-6 were discontinued because IL-6 levels were not observed to increase in MCF-7 cells upon acquisition of resistance to docetaxel. The cytokines CXCL1, CXCL8, and TNF-α (levels presented in figure 3.4, and figure 3.9) were chosen among the remaining cytokines to be the focus of our study based on the volume of literature supporting their role in drug resistance, and the magnitude by which their basal output was increased during acquisition of resistance in figure A5 (below). It is worth noting that the number of adhered cells for each cell line was also presented in figure A5 and shows that during acquisition of resistance to docetaxel there is no detectable increase in the cell number, thus ruling out the effect of cell number on the increase of basal level cytokine release observed.

Multiplex (figure A5)

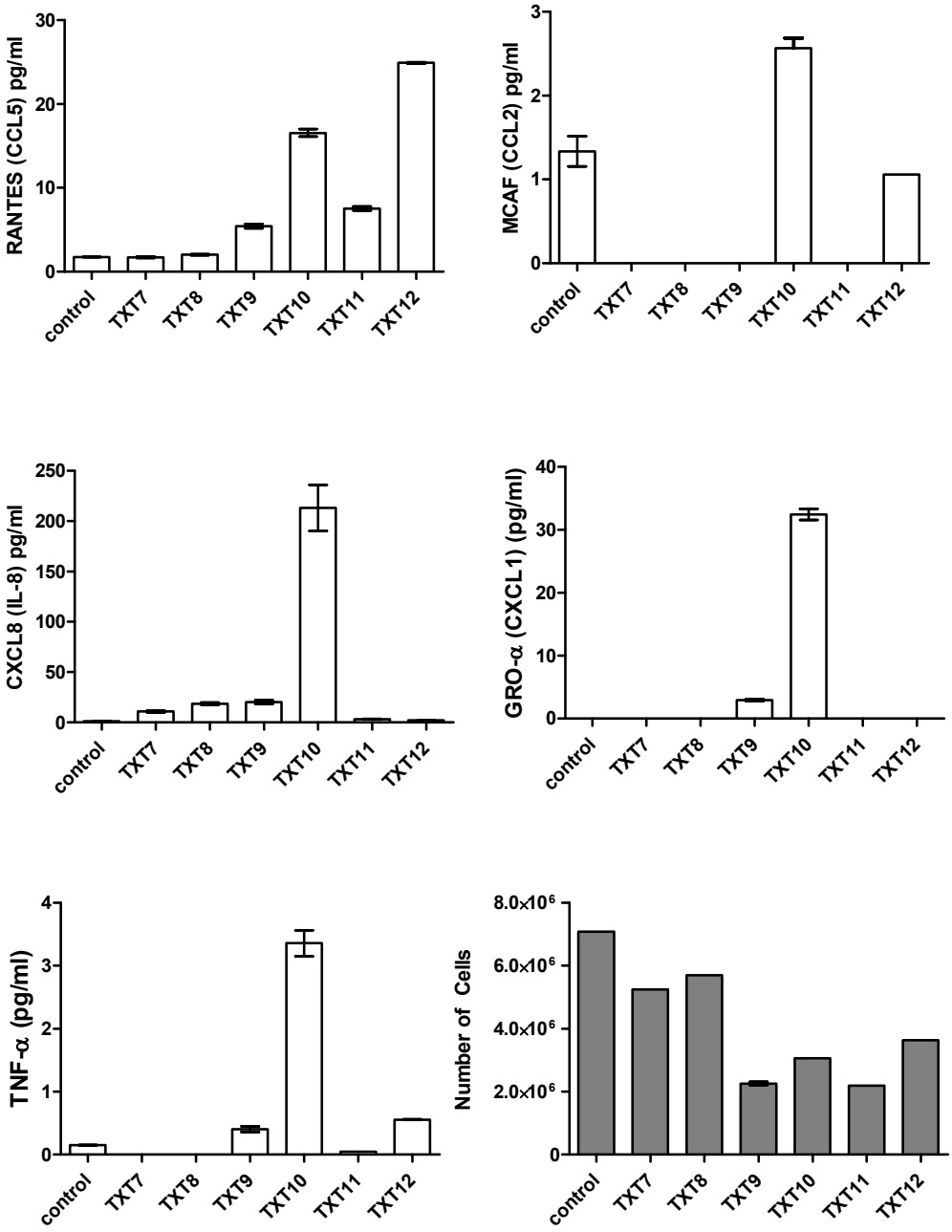

Figure A5 - Level of cytokine in media of various drug-resistant cell lines. MCF-7 co-culture control cells and selection cell lines (MCF-$7_{TXT7\text{-}12}$) were plated for 72 hours, after which media was collected and assessed for levels of cytokine using Bioplex Magpix. N=2 for control and TXT12 cell lines; N=3 for TXT7,8,10, and 11; N=6 for TXT9. Lanes with no values represent media samples containing levels of cytokine below the detection limit of the assay.

CD14 is an LPS-binding protein found at the cell membrane of a variety of cell types and its binding to LPS is suggested to be a requirement for LPS-induced inflammatory effects in human cells (266), including increased TNF-α release. Figure A6 (below) presents data that was generated with the goal of showing that LPS contamination of docetaxel is not responsible for the inflammatory effect observed. The findings presented below suggest that CD14 activity is not a requirement for docetaxel-induced TNF-α release in MDA-MB-231 cells because neutralization of CD14 activity did not have a significant effect on docetaxel's ability to cause TNF-α release. On the otherhand, CD14 neutralization did affect the abillity of LPS to induce the release of TNF-α.

ELISA (figures A6-8)

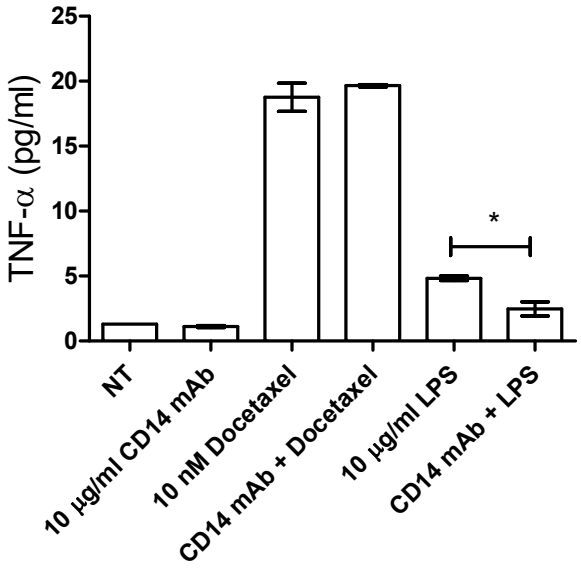

Figure A6 - Effects of a CD14-neutralizing antibody on docetaxel-induced TNF-α release. MDA-MB-231 cells were treated for 72 hours with either docetaxel or LPS and in the presence or absence of a CD14 mAb. Values represent the mean of two replicates. A student t-test was conducted; * p=0.0145

Figure A7 presents data that was generated with the goal of determining whether the ethanol vehicle for docetaxel is responsible for inducing the release of TNF-α from MDA-MB-231 cells. MDA-MB-231 cells were used because they exhibit a strong response to drug, in terms of TNF-α release. The data presented below shows that there was no detectable effect on TNF-α release when an ethanol-only vehicle control was administered to the cells.

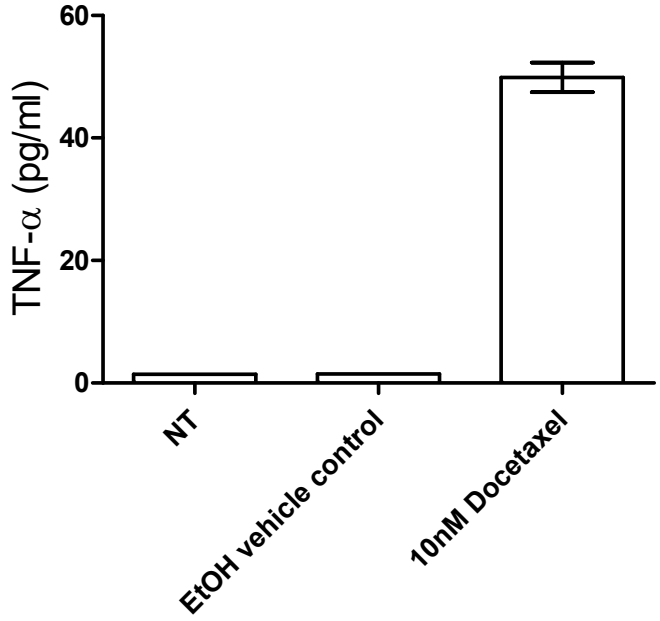

Figure A7 - TNF-α levels in MDA-MB-231 media after treatment with ethanol as a vehicle control. MDA-MB-231 cells were treated for 72 hours with either docetaxel or an equivalent volume of anhydrous ethanol, after which cell media was assessed for TNF-α levels. Values represent the mean of two replicates.

Figure A7 presents data the was generated in order to determine the optimal concentration of lipopolysaccharides (LPS) to treat MDA-MB-231 cells with in order to conduct experimentation that would lead to the generation of figure 3.5 in the results section. The goal was to determine how low of a concentration of LPS could be used, while maintaining the ability to induce TNF-α release from the tumour cells, such that a minimal amount of LPS-RS (TLR4 antagonist) could be used in order to inhibit TLR4 activity.

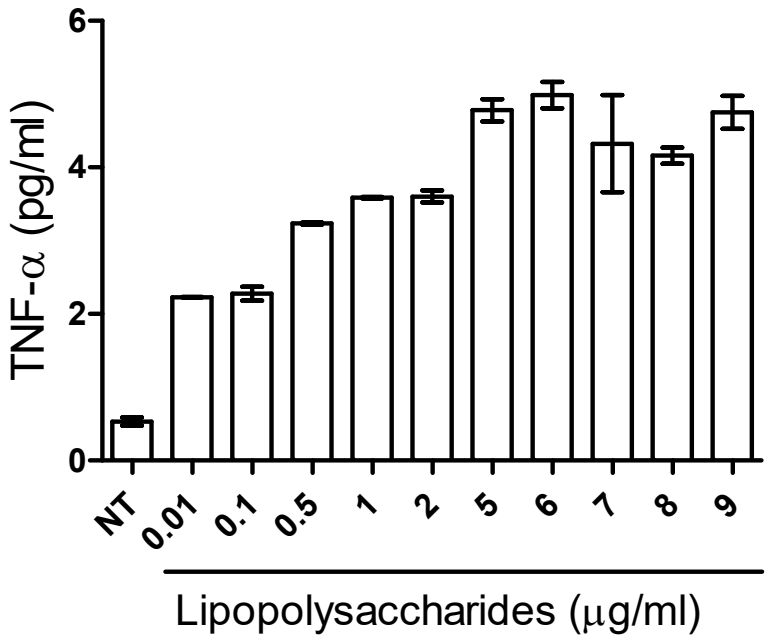

Figure A8 - TNF-α levels in media of MDA-MB-231 cells after treatment with Lipopolysaccharides. MDA-MB-231 cells were treated for 72 hours with a variety of LPS concentrations, after which media was collected and assessed for levels of TNF-α by ELISA. Each value represents the mean of two replicates.